Light-Inducible Molecular Beacons for Spatio-Temporal Activation

and

Visualising the Maturation of microRNA-181 in Neuronal Dendrites

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Jennifer Sarah Rinne
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Dekan: Prof. Dr. Thomas Prisner
Erster Gutachter: Prof. Dr. Alexander Heckel
Zweiter Gutachter: Prof. Dr. Erin Schuman

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If there are no ups and downs in your life, it means you are dead.
Abstract

The establishment of solid phase synthesis within the last decades allowed for the design of highly modified oligonucleotides in all fields of life sciences. Methods like single-molecule real-time tracking of biomolecules like RNA progressed quickly. It became possible to track single RNA molecules from the transcription site within the nucleus to the site of protein biosynthesis within the cytoplasm. However, the detailed mechanism of the nuclear export is still not fully elucidated, even though this is the last quality control point for a mature messengerRNA (abbrev.: mRNA).

Besides, within the last years more and more question arose concerning the local protein biosynthesis of proteins. It was hypothesised that – especially within polar cells like neurons – protein synthesis is not happening ubiquitous within the whole cell. It was suggested that dormant state RNA is transported to the location where the respective protein is needed.

The present PhD Thesis is separated in two main parts. During both projects, molecular tools have been developed to help answering above mentioned scientific questions.

In the first project, a new generation of light-inducible Molecular Beacons has been designed. Molecular Beacons are oligonucleotides that start to fluoresce upon binding to their complementary target sequence of interest. To do so, Molecular Beacons are commonly modified with a fluorophore and a quencher. Regarding published light-inducible designs, fluorescence can only be observed upon target binding, when the Molecular Beacon has been induced by light before.

In contrast to previous designs, the fluorophore of the here presented Molecular Beacon was covalently connected to an additional photocleavable quencher. Like this, the Molecular Beacon can bind to its target sequence without previous light-induction. Only after removal of the second quencher by light, fluorescence can be detected.

Light-inducible Molecular Beacons of the new design achieved signal-to-noise ratios of up to 170:1. An additional advantage is the versatility, since a number of commercially available fluorophore-quencher pairs could be applied. The performance of the Beacon was independent from the synthetic route, whether the fluorophore had been introduced co-synthetically during solid phase synthesis or post-synthetically by covalent modification of functional groups.

However, in vitro tests showed that red-shifted dyes were not suitable due to their hydrophobicity. The resulting stacking and complex formation impaired quenching efficiency.
Abstract

It was not possible to solve this issue during the current PhD Thesis – neither by the usage of light-inducible, fluorescent inorganic nanoparticles – so called quantum dots – nor by red-shifted fluorophores, to which photoremovable protecting groups had been attached that should theoretically result in a quenching effect.

After initial in vitro tests, the best Molecular Beacons were applied in vivo in Chironomus tentans. This organism is the most suitable for single-molecule RNA tracking due to the presence of so-called polytene chromosomes. These structures contain a high copy number of the same gene and the respective mRNA and are easy to localise within the nucleus due to their characteristic structure.

After injection of the Molecular Beacon into the nucleus and subsequent photoinduction, an outstanding signal-to-noise ratio of approx. 80:1 was measured.

Microscopy images of a nucleus of Chironomus tentans before and after light-induction of the new Molecular Beacon design. The black arrow indicates the position of the polytene chromosome.

The aim of the second project was the visualisation of local microRNA maturation in neuronal dendrites. MicroRNA (abbrev.: miRNA) has been shown to be an important cellular tool for post-transcriptional gene regulation. Therefore a molecular probe was designed, that mimicked the native precursor of the respective miRNA – the so-called pre-miRNA. The maturation process, which is mediated by an RNase named Dicer, was supposed to be detectable by fluorescence. For this reason, the pre-miRNA was modified at the enzymatic cleavage position by attaching a fluorophore and a quencher. Upon digestion by Dicer, fluorophore and quencher were separated from each other, allowing fluorescence to be detected. The fluorophore remained at the mature miRNA.

The main challenge of this project was the solid phase synthesis of the long and modified pre-miRNA. Besides, not every tested fluorophore-quencher pair and not every modified position within the sequence were accepted by Dicer. The native cleavage position should be kept untouched despite the presence of bulky modifications.

The usage of Dicer knock-out mice ganglia did not show any fluorescence increase. This experiment was probably the most convincing in vitro negative control.
Principle of the pre-miRNA probe that fluoresces upon Dicer-mediated cleavage. The quencher (Q) is attached to the loop sequence, while the fluorophore (F) remains at the mature miRNA, which is still double-stranded at that time.

In the next step, in vivo experiments were planned. Delivering the probe with a patch pipette was found to be a much better delivery method than common transfection methods for this purpose.

Under basal conditions, there was fluorescence increase in both the soma and the dendrites of the respective neuron. This effect could be increased, when the neuron was depolarised. Generally, the fluorescence signal was much higher in the soma compared to the dendrites. Interestingly, if the important glutamate-dependent NMDA receptors were blocked, the fluorescence value corresponded to the basal level in the case of the soma. Regarding the dendrites, the fluorescence was even below basal levels despite simultaneous depolarisation. These findings indicate an NMDA receptor dependence and consequently a Ca\textsuperscript{2+}-dependent maturation of the respective miRNA.

In further experiments, the pre-miRNA probe was delivered to the neuron and subsequently the dendrites were activated locally by light-inducible glutamate. Within the central nervous system, glutamate is known to be the most important excitatory neurotransmitter. It was observed that at the activated location fluorescence increased, but also dendritic spines started to grow. Sometimes even adjacent spines started to grow in a time-delayed manner. Spines are mushroom-like protuberances along the dendrite, wherever synapses of other neurons have contact.

The figure part a) depicts a time lapse under stimulated conditions. The fluorescence, which arises upon probe processing, arises within 25 min with the highest signal in the soma. Part b) shows the fluorescence signal within a dendritic spine upon local activation of a dendrite.

As a summary, it can be concluded that dendritically localised maturation of the studied miRNA exists. This Dicer-mediated process could successfully be initiated locally by activation of single synapses.
Zusammenfassung


In den letzten Jahren entwickelten sich auch vermehrt Fragen zur lokalen Proteinsynthese. Dabei nimmt man besonders im Fall von polaren Zellen wie Neuronen an, dass die Proteinbiosynthese nicht global im Cytosol stattfindet, sondern es ein Transport der „ruhenden“ RNA bis zu dem Ort geben muss, an dem das entsprechende Protein lokal benötigt wird.

In dieser vorliegenden Arbeit sollen nun in zwei Hauptprojekten molekulare Werkzeuge entwickelt werden, mit deren Hilfe oben genannte Fragestellungen in Zukunft beantwortet werden könnten.


Zusammenfassung

Allgemeines Prinzip eines Molecular Beacons. Der MB liegt als Stamm-Schleifen-Struktur vor, die eine unmittelbare Nähe zwischen Fluorophor (magenta/pink) und Quencher (schwarz) ermöglicht. Somit ist keine Fluoreszenz detektierbar, bis die Bindung an eine komplementäre Target-Sequenz die Stamm-Schleifen-Struktur aufbricht.


Sinnvoller erscheint es, dem Molecular Beacon ein Binden an die komplementäre Target-Sequenz zu erlauben, allerdings die Fluoreszenz erst nach Lichtaktivierung detektierbar zu machen. Ein derartiges Design hat den Vorteil, dass direkt im Anschluss an die Lichtaktivierung eine Fluoreszenzmessung erfolgen kann, da Molecular Beacon und Target-RNA bereits zuvor aneinander gebunden sind.

Dies wurde entsprechend in der vorliegenden Arbeit umgesetzt, indem der Fluorophor mit Hilfe eines zweiten photoabspaltbaren Quenchers verbunden wurde. Dadurch kann der Molecular Beacon an seine Targetsequenz binden, obwohl noch keine Lichtaktivierung stattgefunden hat. Fluoreszenz kann allerdings erst nach photoinduzierter Abspaltung des zusätzlichen Quenchers detektiert werden.


Praktisch zeigte sich bei der Wahl der Fluorophore allerdings die Einschränkung, dass rotverschobene Farbstoffe bzw. deren Quencher, die meist stark hydrophob sind, zu Aggregation neigen und die Quencher dann nicht mehr ausreichend Fluoreszenz-löschend sind. Mögliche Lösungen für dieses Problem wurden in diversen studentischen Arbeiten in Bezug zu dieser Arbeit getestet.


Eine Möglichkeit war die Verwendung eines Chinon-Derivats des Farbstoffes Cyanin 7, der in der Literatur als QCy7 (von engl. quinone Cy7) bekannt ist. Bei diesem Farbstoff durchbricht die Anbringung einer photolabilen Schutzgruppe das push-pull System, sodass keine Fluoreszenz emittiert werden kann. Als problematisch zeigte sich hierbei zum einen die chemische Synthese, zum anderen aber auch die Instabilität in wässrigen Puffern, was eine biologische Anwendung größtenteils unmöglich machte.
Zusammenfassung


Im Cytoplasma werden die prä-miRNAs durch die RNase vom Typ III Dicer in einen ca. 20-nt langen miRNA-Duplex geschnitten. Einer der beiden Stränge ist partiell komplementär zu einer mRNA. Die Bindung der reifen miRNA in Komplex mit diversen Proteinen kann nun die Translation der entsprechenden mRNA auf unterschiedliche Arten unterbinden.

Es wird vermutet, dass vor allem die Dicer-abhängige Reifung der prä-miRNA lokal begrenzt und Aktivitäts-abhängig ist, so wie es auch bei der „bedarfsgeorechten“ Translation von ruhenden mRNA-Molekülen vermutet wird.


Zusammenfassung


Dies lässt darauf schließen, dass die Reifung der untersuchten prä-miRNA in Dendriten von der Aktivität des NMDA-Rezeptors bzw. einem als Konsequenz ansteigenden Ca$^{2+}$-Spiegels in der Zelle abhängig ist. NMDA-Rezeptoren sind wichtige ionotropen Glutamat-Rezeptor in Neuronen, die neben Na$^+$ auch Ca$^{2+}$ in das Neuron passieren lassen.


Es konnte beobachtet werden, wie einerseits Fluoreszenz lokal an der aktivierte Stelle anstieg und gleichzeitig sog. dendritische Spines wuchsen. Zum Teil war auch ein Wachstum benachbarter Spines zu beobachten. Dabei handelt es sich um pilzförmige Aussackungen der Dendriten an Stellen, an denen Vernetzungen zu Synapsen anderer Neuronen existieren.


Als Ergebnis kann geschlussfolgert werden, dass es eine lokale Reifung der untersuchten prä-miRNA durch Dicer in Dendriten existiert. Dieser Prozess kann sehr spezifisch und lokal durch die Aktivierung einzelner synaptischer Verbindungen initiiert werden.
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Introduction

In the following PhD Thesis, the research is directed towards the chemical synthesis of modified oligonucleotides for the use in two different biological in vivo assays. This study will first introduce new strategies for improved single-molecule real-time imaging of intracellular mRNA traces. A second project aims at the visualisation of molecular processes in neuronal cells during learning and memory formation.

All these probes are highly chemically modified at well-chosen positions of the oligonucleotide so that the synthesis can exclusively be performed using chemical solid phase synthesis.

This technique will first be outlined. Afterwards biochemical basics, the research aim, and results will be discussed.

Solid Phase Synthesis of Oligonucleotides

For a long time, the chemical synthesis of oligonucleotides has been very challenging. Especially as the need for chemically highly modified and long oligonucleotides rises, solid phase synthesis (SPS) has become the method of choice. By now, various modifications for different applications are commercially available. The most important of these modifications – in particular dye-labelled oligonucleotides – will be discussed later in more detail.

ALEXANDER TODD and his group were the first ones who succeeded in synthesising a naturally linked dT-nucleotide in 1953.¹ In a first step, he produced an H-phosphonate that later reacted to a phosphotriester. For the first time, protecting groups (PGs) such as acetyl and benzyol groups were used to prevent free hydroxyl groups from being linked in an unintended way. Like this, TODD et al. could selectively produce a covalent 3’→5’-connection.¹⁻³

![Figure 1: Synthesis of a natural desoxy-thymidine dimer. An initially produced benzoyl-protected H-phosphonate is activated by N-chloro succinimide and is then coupled to the second 3’-acetyl protected thymidine.¹](image-url)
Interestingly, a related method – the *H-phosphonate* method – is still of importance to date. In this method, a nucleoside with a 3’-H-phosphonate is coupled to the free 5’-hydroxyl group of a second nucleoside (cf. Figure 2). The final phosphor oxidation can be conducted after the synthesis to produce the natural phosphate backbone. In contrast to the method of Tødd et al., no additional protecting groups at the H-phosphonate are necessary. Especially the benzoyl group used by Tødd et al. was rather difficult to remove. A number of condensation agents (e.g. acid chlorides) has been tested and optimised, but undesired side reactions such as acylation reactions could not be fully abandoned. Apart from that, the reaction yields are still inferior to the currently most used phosphoramidite method explained below.

Figure 2: Solid phase H-phosphonate method. This method is still used to date and closely related to the H-phosphonate dimer synthesis by Tødd et al. In this method, an H-phosphonate is coupled to a free 5’-hydroxyl group of a solid-phase bound nucleoside. The final oxidation step produces the natural phosphate linkage.

In 1956, Khorana et al. published an alternative approach that is also known as *phosphodiester synthesis*. Here, 3’-acetyl protected 5’-phosphate nucleotides were coupled to a 3’-hydroxyl group. This reaction needs to be activated by a condensation agent like DCC or TsCl (abbrev.: dicyclocarbodiimide, 4-toluenesulfonyl chloride). Upon base-catalysed removal of the 3’-acetyl group, further chain elongation becomes possible. These reactions show yields of approx. 50-70% per coupling, a fact that still limits this methods to short sequences. Even though this technique is by far not as selective as the one described by Tødd et al. three years earlier, it circumvents the necessity of a benzoyl-protected phosphor species, which was difficult to deprotect. However, the abdication of a PG caused some side reactions such as branched pyrophosphate oligomers. Nevertheless, Khorana’s work had a major impact on the elucidation of the genetic code. In particular the fact that he was the first one who used protective groups for the exocyclic amino groups of the nucleobases allowed him to use all nucleotides and not to be restricted to dT.
In the 1960s, \textsc{letsinger and reese} further developed the phosphotriester approach of \textsc{todd} et al. By then, more suitable PGs for the phosphor species were accessible, i.e. the 2-cyanoethyl group. The reduction of side reactions such as branching massively increased synthesis yields and in parallel decreased the reaction time.\textsuperscript{11–13} While all other internucleotide coupling reactions had been conducted in solution, the phosphotriester method was also implemented on polystyrene beads and marks the invention of solid phase synthesis.\textsuperscript{14}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Phosphotriester approach by \textsc{letsinger and reese}. A 5'-protected 3'-phosphate is coupled to a free 5'-hydroxyl group of a 3'-protected nucleoside. The reaction is activated by TPSCI (abbrev.: 2,4,6-triisopropylbenzenesulfonyl chloride). Branching and pyrophosphate formation are prevented by the usage of the cyanoethyl group as phosphate protecting group.\textsuperscript{11,12}}
\end{figure}

In 1976, \textsc{letsinger} together with \textsc{lunsford} showed that coupling yields could be further increased by using more reactive P(III)-species, i.e. 3-O-chlorophosphites instead of the common P(V)-species.\textsuperscript{15,16}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5}
\caption{In the 1970s, \textsc{letsinger and lunsford} were able to dramatically increase the yield of internucleoside linkages by using substantially more reactive P(III)-derivatives such as 3'-O-chlorophosphites.\textsuperscript{16}}
\end{figure}
Finally, Caruthers and Beaucage replaced the reactive P(III)-3-O-chlorophosphite with another P(III)-species, the so-called phosphoramidite that is easier to handle, as it is less sensitive to hydrolysis. They finally established 2-cyanoethyl as a more easily removable phosphor protecting group. To date, SPS is still conducted in this way.17,18

Figure 6: Caruthers et al. developed more stable, but still reactive P(III)-phosphoramidites. Even to date, diisopropyl amine derivatives are widely accepted due to their higher stability compared to dimethyl amine amidites.19 It was also shown, that the 2-cyanoethyl is the most suitable phosphoramidite protecting group, as it is easily removed during basic deprotection of exocyclic amino groups of the oligonucleotide.17

With this reliable, well yielding coupling reaction, DNA synthesis could finally be adapted on Merrifield’s solid phase synthesis for peptide synthesis. The synthesis of oligomers on a solid phase is clearly advantageous: It is less time-consuming, because intermediate purification steps are not necessary. Reactants can be used in high excess, a fact that further improves yields.20

Regarding suitable solid phases, it should be noted that inert, non-swelling materials must be chosen. To date, mostly controlled pore glass (CPG)21 is routinely used as well as specially cross-linked polystyrene (PS) materials22. Depending on the desired length of the oligonucleotide, CPG material is available with different pore sizes (500 Å for up to 60mers and 1000 Å for up to 150mers, respectively). Most solid phase materials are already coupled to the first nucleoside via its 3’-hydroxyl group. The 5’-hydroxyl group is protected by the dimethoxytrityl (DMTr) PG, as it is commonly done for phosphoramidites. It has been shown that the distance of the first nucleoside to the solid support is very critical. Most CPG materials are linked via long chain amino acids (lcaa) to a shorter succinyl linker and finally coupled to the 3’-hydroxyl group of any commercially available 3’-terminal modification. For PS materials the lcaa linker is usually substituted by an amino methyl group.23,24
Figure 7: As solid support for SPS, controlled pore glass (CPG) or polystyrene (PS) is mostly used. The material can vary in linker length and pore size, the latter being a limiting factor for oligonucleotide length. The 3’-modification is either covalently linked to the solid phase via long chain amino acid (Icaa) or amino methyl linkers. An ester group causes base lability.24

It is worth mentioning, that so called universal supports have been published a couple of years ago and have become commercially available more recently. In this case, a non-nucleoside linker is attached to the solid phase and the first nucleoside or its derivative, respectively, is introduced during the first synthesis cycle. An important advantage of the universal supports is the possibility to introduce phosphoramidites that are not commercially available as solid supports at the 3’-end of an oligonucleotide.25,26

Synthetic Cycle

The oligonucleotide SPS using the phosphoramidite method is a cyclic mechanism. Every phosphoramidite is coupled to the solid support by four reactions, which are repeated for every phosphoramidite of the oligonucleotide sequence. The single steps of this cycle are defined below in more detail.

Worth mentioning, the oligonucleotide SPS is carried out in the direction from 3’-end to 5’-end (3’→5’). Accordingly, the oligonucleotide is linked to the solid support via its 3’-hydroxyl group. Consequently, the chemical synthesis direction is in contrast to the enzymatic in vivo synthesis that starts at the 5’-end (5’→3’).

Detritylation

Every phosphoramidite – even the nucleoside coupled to the solid support – bears a dimethoxytrityl (DMTr) protecting group at its 5’-end. This acid-labile PG is removed at the synthesiser by 3% trichloro acetic acid (TCA) or trifluoro acetic acid (TFA) in methylene chloride. The cleaved DMTr cation is flushed from the solid support and can be used to determine the yield of each cycle, as the amount of DMTr cation can be measured by UV/Vis absorption measurements due to its orange colour or by conductance measurements. The free hydroxyl group at the 5’-end can then be used for strand elongation. Importantly, the
deprotection period must be carefully chosen. It is widely known that the nucleosides guanosine and adenine are depurinated in the presence of strong acids.\(^\text{27}\)

\[ \text{Figure 8: In the first step, the acid labile DMTr protecting group is removed from the 3'-end using 3\% trichloro acetic acid (TCA) in methylene chloride.} \]

**Coupling**

The coupling of the phosphoramidite with the free 5'-hydroxyl group is a catalysed reaction. Tetrazole derivatives are mostly used as catalysators, e.g. ethyl thiotetrazole (ETT) or benzyl thiotetrazole (BTT). They are both nucleophilic and acidic with BTT having even a slightly smaller pK\(_a\) (4.1 compared to 4.3 for ETT). Some studies propose that the acidic character of tetrazole based activators may lead to detritylation of the phosphoramidite, causing side reactions and the formation of elongated (n+1)-products. For this reason, it is sometimes suggested to use 4,5-dicyanoimidazole instead, which is more nucleophilic with a much higher pK\(_a\) value (5.2).\(^\text{28,29}\)

First, the N,N-diisopropyl amino group of the phosphoramidite is protonated, so that its leaving group characteristics are increased. The protonated N,N-diisopropyl group is then substituted by the activator forming a tetrazolide. Finally, the free 5'-hydroxyl group at the solid support attacks the phosphor and replaces the tetrazole derivative in a nucleophilic substitution reaction.\(^\text{6,23,30}\)
Figure 9: Formation of the internucleoside linkage can only occur in the presence of an activator such as tetrazole derivatives like ethyl thiotetrazole (ETT, a), benzyl thiotetrazole (BTT, b), or 4,5-dicyanoimidazole (c). The activator replaces the N,N-diisopropyl amino group and allows for the nucleophilic attack of the 5’-terminal hydroxyl group (d).

**Capping**

The third reaction within the cycle is called *capping*. Free 5’-hydroxyl groups, which have not successfully been coupled to a phosphoramidite during the previous step, need to be masked. Otherwise, the free OH-group would react during following cycles and produce oligomers with internal base deletion (⟨n-1⟩-products) that are hardly separable during final purification steps. For this reason, free hydroxyl groups are acylated usually by acetic anhydride (Ac₂O) or phenoxycetic anhydride (Pac₂O). This reaction needs base catalysis, usually N-methylimidazole is used.

Figure 10: The next step is called capping and prevents the formation of ⟨n-1⟩-oligomers. By acetylation of unreacted, free 5’-OH-groups, a further reaction and elongation of these truncation sequences is avoided.
Oxidation

After coupling, the P(III)-triester linkage does not correspond to the natural P(V)-phosphate backbone. Additionally, this linkage is much more unstable regarding hydrolysis. Therefore, a final oxidation step is necessary. In this case, a mixture of iodine, water, and a weak base, mostly pyridine, is used as oxidising agent to obtain the P(V)-phosphotriester.

Figure 11: In the last step of the cycle, the P(III)-linkage is oxidised using iodine in pyridine to yield the natural P(V)-phosphate linkage.

Afterwards, the cycle can be repeated for every nucleotide desired in the sequence. For the last 5'-terminal phosphoramidite within the sequence, it is common practice not to remove the final 5'-DMTr protecting group. This PG increases hydrophobicity of the oligomer, so that it is easier to purify via reversed phase (RP-) HPLC, as it elutes later compared to truncation sequences without DMTr group.
Figure 12: Synthesis cycle of solid phase oligonucleotide synthesis using the phosphoramidite method. The first nucleoside is coupled to the solid support at its 3'-end. For this reason, the 5'-hydroxyl group is protected with the acid labile dimethoxy trityl (DMTr) group. 1. This protecting group is initially removed under acidic conditions e.g. by TFA. 2a. Afterwards, the next nucleoside phosphoramidite is activated by tetrazole derivatives. 2b. The activated phosphoramidite is then flushed on the solid support and coupled to the deprotected 5'-hydroxyl group of the previous nucleoside. 3. Nucleosides that have not been coupled successfully are capped using acetic acid anhydride (Ac₂O) to avoid (n-1)-truncation oligomers. 4. The P(III)-species is finally oxidised using iodine to afford the natural P(V)-phosphate linkage.

**Deprotection and Purification of Chemically Synthesised Oligonucleotides**

After solid phase synthesis, the oligonucleotide must be cleaved from the solid support. Apart from that, the protecting groups of exocyclic functionalities at the bases as well as the cyanoethyl group at the phosphate need to be removed. For that, usually aqueous ammonia or a solution of ammonia and methylamine (1:1 (v/v), AMA) is used.

First, the oligonucleotide is cleaved from the solid support by aminolytic cleavage of the succinyl linkage. Secondly, the cyanoethyl group is removed from the phosphotriester by β-elimination reaction. However, the rate-determining step is the removal of base-labile protecting groups from the nucleobases. To date, there are different protecting groups available that reduce the necessary basic incubation time as much as possible.\textsuperscript{31,32}
Solid Phase Synthesis of Oligonucleotides

Figure 13: After SPS, the oligonucleotide is cleaved from the solid support using basic conditions such as aqueous ammonia or methylamine. During this procedure, the cyanoethyl group is removed in a β-elimination step (a). The diverse protecting groups of the exocyclic amino groups are cleaved off as well. The cleavage of the latter ones is the rate-determining step. Figure part (b) depicts a number of different protecting groups with decreasing deprotection times.

Modifications that are introduced into the oligomer via solid phase synthesis may have special cleavage conditions, as some may degrade under harsh basic conditions. Therefore, the combination of different protecting groups must be carefully considered.

In contrast to the solid phase synthesis of DNA, the SPS of RNA oligomers is more sophisticated due to the presence of an additional hydroxyl group at the 2'-end. To avoid side reactions during solid phase synthesis, this free hydroxyl group needs to be protected as well. It is necessary to protect the 2'-OH-group orthogonally to the above mentioned base-labile PGs, as RNA tends to be cleaved under basic conditions in a cyclic mechanism (cf. Figure 14).

Figure 14: During RNA deprotection, it is important that the 2'-OH protecting group is still intact during basic cleavage condition. When incubated with base, RNA is degraded due to the formation of a stable, entropically favoured five-membered phosphate ring.
Introduction

Routinely, there are two 2'-hydroxyl protecting groups available that are both fluoride-labile: the tert-butyldimethoxysilyl (tBDMS) and triisopropylsilyloxymethyl (TOM) protecting group. While tBDMS-protected RNA phosphoramidites are commercially available, TOM-protected phosphoramidites are still under patent. Nevertheless, some studies have been shown that TOM-protected RNA phosphoramidites tend to produce higher yields, especially for long oligonucleotides (>50mer), most likely due to the decreased steric hindrance (cf. Figure 15).33,34

A completely new approach is the so-called 2'-thiomorpholine-4-carbothioate (TC) strategy that is proposed to be of special interest for syntheses of up to 100mers in a massively reduced reaction time. Additionally, base labile groups as well as the TC protecting group can be deprotected in a single step using anhydrous ethylenediamine.35

![Figure 15: Three different 2'-OH protecting groups are commonly used during RNA solid phase synthesis. From left to right: tert-butyldimethoxyisilyl (tBDMS), triisopropylsilyloxymethyl (TOM) and the rather new 2'-thiomorpholine-4-carbothioate (TC) protecting group.](image)

After synthesis and deprotection, the oligonucleotide needs to be purified to separate the full-length target sequence from truncations. Generally for DNA, oligomers can be isolated via RP-HPLC, especially if they still bear the final 5'-DMTr group, as mentioned above. The DMTr group can then be removed by treatment with 80% acetic acid for 20 to 30 min. As RNA is generally more polar, the commonly chosen method is anion exchange (AE) chromatography followed by desalination.

Synthetic Modification of Oligonucleotides

The establishment of oligonucleotide synthesis allows for the design of highly modified oligonucleotides, which have become of great interest for the last decades. Especially the design of fluorescently labelled oligomers is important for advancing microscopy techniques. Suitable labels need to fulfil certain requirements. First, they should be easy and mild to attach. Secondly, most labels are not supposed to affect the structure of the oligonucleotide, even though there are some cases in which forced structural rearrangement is desired, i.e. photolabile protecting groups (see below). Additionally, it is also important that modifications can resist reaction and application conditions. It is, for instance, common practice to heat oligonucleotides to up to 95 °C to hybridise single strands. The most important labelling strategies as well as their assets and drawbacks, respectively, will be discussed in this subchapter.
Commercially Available Phosphoramidites

The easiest way to introduce modifications into a desired oligonucleotide sequence is the usage of commercially available phosphoramidites. This is clearly the least time consuming method for the user. Critical steps during the SPS such as coupling time, resistance against SPS chemicals, and cleavage conditions have already been tested and optimised by the manufacturer.

Certainly, the most important drawback is the fact that some elaborate modifications are expensive. Besides, the user is restricted to the available modifications in terms of linker length or modification sites at nucleobases for instance. Some preferable labels might never be available as phosphoramidites, as the molecules are not resistant against SPS chemistry. Among the commercially available phosphoramidites, there are (fluorescent) dyes, purification labels such as biotin, radioisotope labelled phosphoramidites as well as a number of functionalities for later individual labelling such as amino, thiol groups, and alkynes. Some of them are depicted in Figure 16.

![Figure 16: The figure shows a small number of commercially available phosphoramidites for the modification of oligonucleotides. Most of them have been used during this thesis. a) internal Fluorescein modification, b) TAMRA-dT, c) Pyrene-dU, d) BHQ2 dark quencher for 5’-terminal modification, e) internal alkyne linker for post-synthetic click chemistry modification, f) photocleavable linker.](image)

Importantly, if several modifications are combined within an oligomer, it must be carefully considered, whether these labels are compatible with each other, for instance in terms of cleavage conditions.

Worth mentioning, oligonucleotides that have been synthesised by SPS do not bear a 5’-phosphate, as enzymatically produced DNA/RNA does. To mimic natural structures, it is sometimes necessary to add an additional phosphate moiety at either end of the oligomer. Apart from enzymatic approaches, which will be discussed later in this chapter, this can also be conducted during solid phase synthesis using modifications, as shown in Figure 17.
Introduction

One additional interesting modification is the synthesis of oligonucleotide phosphorothioates. The replacement of one O-atom in the phosphate moiety by sulphur is of special interest regarding increased hydrolysis and exonuclease resistance. This way of protecting oligomers from being degraded within a cell is routinely used in RNA interference experiments. For preparation, a so-called Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-Dioxide) replaces the oxidising reagent during SPS. However, the presence of sulphur at either position of the phosphate causes an additional chirality centre, which makes purification a challenge, especially if several phosphorothioates have been inserted. It has also been shown that too many sulphur atoms within the oligonucleotide backbone have cytotoxic effects.

To avoid endonuclease degradation of chemically synthesised probes, there are also some nuclease resistant DNA/RNA derivatives available. The most prominent among them are 2'-O-methyl-RNA, 2’-F-DNA, and so-called locked nucleic acid (LNA). Especially 2'-O-methyl-RNA and LNA are of importance, as their binding thermodynamics are much higher compared to DNA and RNA themselves. Depending on the desired application, this might have several additional advantages. The probes can be much shorter showing the same binding behaviour compared to longer unmodified complementary strands. Natural counter strands can be replaced irreversibly and some enzyme activities can be fully suppressed. For LNA this behaviour is due to the forced helical structure caused by the methylene bridge.
Chemical Synthesis of Modified Phosphoramidites Using the Example of Photolabile Protecting Groups

For modifications that are not commercially available and that cannot be applied post-synthetically it is still possible to chemically synthesise individually modified phosphoramidites. This allows for flexibility and e.g. linker lengths or modification sites can be chosen according to individual application plans. A major drawback is clearly the elaborate chemical synthesis, as phosphoramidite modifications are usually multistep reactions making this strategy the most time consuming one. Especially if nucleobases are derivatised, the synthesis must be conducted for every of the four nucleobases. Besides, it is sometimes hard to foresee, whether the synthesised compound will be stable against all SPS reagents as well as resistant under basic cleavage conditions. Additionally, suitable solvent mixtures and coupling times must be optimised, before the phosphoramidite can be applied routinely.

One example of individually synthesised phosphoramidites, which also play an important role in this study, are photolabile protecting groups (PPG) sometimes also referred to as caging groups. Caging groups were originally supposed to act as additional orthogonal protecting groups during organic synthesis. To date, several studies have proven their importance to elucidate biochemical and physiological processes on a molecular level. Caged compounds are mostly biological molecules that have been inactivated by the covalent binding to a cage. The inactivity can be caused by the blockage of biologically active centres due to steric hindrance. Upon irradiation with light of a certain wavelength, the PPG is removed irreversibly and the biological activity is regained. There are various advantages for light as triggering signal. Depending on the chosen energy and wavelength, respectively, light is not harmful to living organisms. Solely light within the UV C/B range (200-315 nm) is absorbed by nucleobases causing photoreactions and destruction. In particular for DNA, some UV-light catalysed photoreactions such as dCdC-dimer formation cause mutations and is one of the main causes for skin cancer. In contrast, red-shifted wavelengths are not absorbed by biologically relevant chromophores and can therefore be seen as non-toxic.

Figure 20 depicts some well-established photolabile protecting groups. These can be based on o-nitrobenzyl- (NB), o-nitrobenzofuran (NDBF), coumarin-moieties etc.
Particularly for biological applications, PPGs must meet some special demands:

- High absorption coefficient, so that most photons are absorbed by the PPG
- High quantum yield (φ), so that most absorbed photons are used for the photoreaction
- Fast photoreaction kinetics
- Intoxic cleavage products
- The cleavage products should have an altered absorption spectrum in order not to compete with the uncleaved PPGs

Photochemical data of photolabile protecting groups cannot easily be discussed, as values such as absorption maxima strongly depend on the chromatic shift of substituents or even the polarity of solvents. Comparably, the cleavage efficiency is dependent on the leaving groups. As one would expect, good leaving groups such as phosphate increase quantum yields compared to amines in consideration of the solvent.\textsuperscript{45}

In this study, solely o-nitrobenzyl (NB) derivatives have been used, i.e. o-nitrophenylethyl (NPE) and o-nitrophenylpropyl (NPP). Both groups can be removed by irradiation with 365 nm light. While the cleavage product of NPP is not supposed to be toxic, as it does not bear a potentially toxic nitroso group, the cleavage mechanism might lead to pH-dependent side reactions that would cause incomplete deprotection and consequently irreversible inactivity of the biological compound. The cleavage mechanisms for NPE and NPP are shown in Figure 21 and 22.\textsuperscript{46–48}
**Figure 21:** Mechanism of photolysis for ortho-nitrobenzyl (NB) and its derivatives such as ortho-nitrophenylethyl (NPE). Photon absorption causes a 1,5-proton migration yielding into the so-called aci-nitro tautomer. In a next step the oxygen atom of the nitro group can act as a nucleophile and form a dicyclic benzisoxazolidine. The further resulting hemiacetal can then eliminate the leaving group ROH under formation of an aromatic nitroso-aldehyde moiety.\(^{16}\)

**Figure 22:** The cleavage mechanism of ortho-nitrophenylpropyl (NPP) slightly differs from the NB photolysis. In contrast to NB, the formation of the dicyclic benzisoxazolidine causes an irreversible side product under acidic conditions. At higher pH values, the deprotonated aci-nitro moiety is able to eliminate the leaving group RO\(^-\). The remaining nitro-styrene product is less toxic compared to most nitroso-compounds.\(^{16}\)

*ortho*-Nitrobenzyl was first used by Engel et al. to cage cAMP (abbrev.: cyclic adenosine monophosphate) at its phosphate moiety.\(^{49}\) In contrast, Heckel et al. applied caging groups on nucleobases to disrupt Watson-Crick-base pairing.\(^{50}\) However, a study by Schäfer et al. suggests that the main destabilising effect is the disruption of neighbouring nucleobases regarding \(\pi\)-stacking.\(^{51}\)
Introduction

Figure 23: Different approaches for the photolabile protection of (oligo-)nucleotides. ENGELS et al. caged a cAMP by covalently attaching a photolabile protecting group to the phosphate. In contrast, HECKEL et al. used PPGs to prevent the binding of an oligonucleotide to its complementary strand. Two studies by RODRIGUES et al. and SCHÄFER et al. have shown that PPGs, which have been attached to nucleobases, disturb both Watson-Crick base pairing as well as base stacking.49-51

Worth mentioning, some PPGs are also able to perform two-photon-uncaging. Two-photon uncaging is based on the concept that two photons of half the energy that is usually needed can be absorbed almost simultaneously. This is only possible, when a coherent laser beam is focused. Thus, the two-photon uncaging process offers a three-dimensional, spatially localised way to activate biomolecules. Especially big chromophores with a wide-spread π-system and a “push-pull mechanism” by the substituents, as it is found in NDBF or DEACM, can be removed by IR light.52

Post-Synthetic Labelling

Labels can also be introduced into an oligonucleotide after SPS. In this case, functionalities such as amino, thiol, or alkyne groups need to be introduced during SPS. After synthesis, the oligomer can either be labelled directly on the solid support or after cleavage and purification in solution. The latter has the advantage that truncation products, which might also bear the labelling functionality, have already been removed. Like this, side reaction products that are hard to purify do not occur. If several modifications need to be introduced into an oligomer, it is advantageous that the according labelling reactions are orthogonal to each other. The most important labelling reactions are depicted in Figure 24. Among them, there are electrophilic addition of a thiol group on a maleimide, coupling of active esters (e.g. N-succinyl hydroxid (NHS)) with amino groups, and the copper(I)-catalysed Huisgen-like click reaction.
Synthetic Modification of Oligonucleotides

Figure 24: The figure depicts four different post-synthetic labelling strategies of modified oligonucleotides. a) and b) show the labelling of amino groups using either NHS active esters or cyanates. In both cases, the pH needs to be above 8.0, since at lower pH values, aliphatic amino groups are not nucleophilic enough. In contrast, under extreme basic conditions hydroxide ions from aqueous media might react instead. At lower pH values, thiol groups can successfully react with maleimides (c) even in the presence of amino groups. Above pH 8.0 aliphatic amines are able to react in an equivalent way. A fully orthogonal labelling strategy is the copper(I)-catalysed Huisgen-like click reaction depicted in d). The mechanism is discussed later.

One of the main advantages is the fact that most of these labelling reactions form biochemically stable covalent bonds such as thiourea linkages and triazole groups. Especially if the desired modification is commercially available as labelling reagent, these methods are very straightforward and usually optimised by the manufacturer. Otherwise, modifications can be attached to the functionalised oligonucleotide by routinely used chemical coupling reactions using carboxylic acids (cf. Figure 25).

Figure 25: The formation of an amide using any carbodiimide – in this case the reaction with dicyclohexylcarbodiimide (DCC) is shown. An acid will react with the carbodiimide to produce the intermediate O-acylisourea, which can be compared to other active esters. The O-acylisourea is already able to react with amines to give the desired amide. Alternatively, the reaction efficiency can be further increased by replacing the DCC intermediate with hydroxylbenzotriazole (abbrev.: HOBt), as it is known from peptide synthesis.\textsuperscript{23}

\begin{center}
\begin{tikzpicture}
\node [draw, rectangle, text width=\textwidth] {
\begin{minipage}{\textwidth}
\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure25}
\caption{The formation of an amide using any carbodiimide - in this case the reaction with dicyclohexylcarbodiimide (DCC) is shown. An acid will react with the carbodiimide to produce the intermediate O-acylisourea, which can be compared to other active esters. The O-acylisourea is already able to react with amines to give the desired amide. Alternatively, the reaction efficiency can be further increased by replacing the DCC intermediate with hydroxylbenzotriazole (abbrev.: HOBt), as it is known from peptide synthesis.\textsuperscript{23}}
\end{figure}
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\end{tikzpicture}
\end{center}
In this study, mostly amino group labelling using NHS active esters and the Cu(I)-catalysed azide/alkyne cycloaddition (CuAAC) have been applied. The amino group labelling is a well-established method and a lot of amino functionalised phosphoramidites are available. Usually this labelling procedure does not cause side reactions, as the exocyclic amino groups at the nucleobases are not nucleophilic enough. In recent times, new active esters have been developed that are more stable against hydrolysis. These alternative compounds such as tetrafluorophenyl (TFP) and sulfodichlorophenol (SDP) esters allow for longer reaction times, even at elevated temperatures, and are therefore supposed to improve labelling yields.\textsuperscript{54}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure26.png}
\caption{a) The figure depicts two examples of amino modified phosphoramidites. The amino modification can either be attached to the nucleobase or an additional linker can be attached to the end of an oligonucleotide. b) Three examples of commercially available active esters. The most common, but also most instable one is NHS (N-hydroxysuccinimide). Some AlexaFluor dyes are available as more stable active esters such as SDP (sulfodichlorophenol) or TFP (tetrafluorophenol). These active esters can even be heated and are stable under basic conditions for several hours.}
\end{figure}

In recent years, the Cu(I)-catalysed azide/alkyne cycloaddition (CuAAC) has become the most popular click reaction in biochemical applications. This reaction is easy to handle, bio-orthogonal, forms biologically stable bonds and is widely applicable. Worth noting, it is regioselective forming exclusively 1,4-disubstituted triazoles. Additionally, the reaction is orthogonal to most other labelling reactions and the presence of a variety of functionalities is accepted. Especially for biological systems, the fact that the reaction can be conducted in aqueous buffered media is very advantageous. The Cu(I) ligand tris(benzyltriazolylmethyl)amine (TBTA) makes the reaction less oxygen-sensitive, as it prevents the Cu(I) from being oxidised. New compounds also allow for orthogonal di-labelling, as trimethylsilyl (TMS) protected alkyne groups do not react in the CuAAC reaction until removal of the PG.\textsuperscript{55}
Synthetic Modification of Oligonucleotides

Figure 27: a) Different nucleosidic alkyne modifications for the post-synthetic Huisgen-like click reaction. The alkyne group can be silyl-protected to allow for the attachment of two different labels, as the protected alkyne does not react.

b) Reaction cycle of Huisgen-like Cu(I)-catalysed azide/alkyne cycloaddition (CuAAC). The copper(I) species forms a π-complex with the triple bond of a terminal alkyne. Studies have shown that the pKₐ of this complex is increased, deprotonation can take place, and a Cu-acetylide intermediate is formed. Afterwards the azide displaces one ligand to generate a copper-azide-acetylide complex and allow for cyclisation. This is followed by protonation. The product is formed by dissociation and the catalyst-ligand complex is regenerated for further reaction cycles.⁵⁶

There is also the possibility to label functionalised oligonucleotides directly after SPS on the solid support. This approach is of special interest, if long oligonucleotides with strong secondary structures must be labelled, as in these cases yields of common post-synthetic labelling strategies are usually low.⁵⁷

In the case of amino groups, the protecting group of this amino group must be carefully chosen. There are PGs available that can be removed under mild basic conditions without cleaving the oligomer from the solid phase. One example is the Fmoc-amino PG that can be deprotected using 10% piperidine (v/v) in DMF. For a long time, the Fmoc group was not the preferred protecting group. Deprotection trials caused alkylation reaction by the released acrylonitrile. This issue can be overcome by previously removing the cyanoethyl group using disopropylamine (1% (v/v)) in acetonitrile.⁵⁸

However, for this reason CuAAC reactions are very useful for labelling on the solid phase, as alkyne groups are usually unprotected during SPS. Importantly, the label needs to be compatible with basic and fluoride-dependent cleavage conditions, if it is installed on the fully protected oligomer on the solid phase.
Enzymatic Modifications of Oligonucleotides

Apart from chemical modifications, it is also possible to modify oligomers enzymatically. The most prominent example is the T4-polynucleotide kinase that can be used to attach phosphate moieties at the 5'-hydroxyl group of double or single stranded oligonucleotides. This is especially useful for radioisotope labelling of oligomers with $\gamma$-$^{32}$P-ATP. However, usually enzymatic phosphorylation shows lower yields compared to the usage of chemical phosphorylation reagents.

Another approach, which is still conducted enzymatically to date, is the ligation of oligomers. Here, oligonucleotides, which are too long for SPS (>150mer) and modified, so that they cannot be synthesised biologically, can be isolated. One routinely used protocol of RNA ligation uses a DNA splint to prevent secondary structures and to keep both ends close to each other. Importantly, T4-ligases need 5'-phosphate groups for ligation. The position for ligation needs to be considered carefully, as not all 3'-hydroxyl groups and 5'-phosphate groups can be ligated with the same efficiency. The basic mechanism of ligation is depicted in Figure 29.

Figure 28: Fmoc-protected amino-dT phosphoramidite. The Fmoc group can be deprotected using 10% piperidine (v/v). Under this basic condition the oligonucleotide is not cleaved from the solid support and most base-labile protection groups stay intact.
Figure 29: Template-mediated ligation of RNA. The two RNA strands (black) are hybridised with a complementary DNA (blue, splint DNA). Importantly, the 5’-end of one RNA strand needs to bear a phosphate. To avoid cyclisation, the 3’-hydroxyl group of this strand might be masked by a protecting group. The T4 ligase is able to close the gap within the phosphate backbone. Worth mentioning, not every base is ligated with the same efficiency. Ligation works best with adenosine as the 3’-base, while cytidine seems to be the best one at the 5’-phosphate strand.59
Molecular Beacons

With the establishment of solid phase oligonucleotide synthesis, highly-modified oligomers, which can easily be labelled in high yields, have become available for a wide range of applications in biochemistry, material science, and nanoarchitecture. One of the biological possibilities is the _in vivo_ labelling of cellular components and their analysis using microscopy techniques. One technique that has become very popular for the last couple of years is the **Molecular Beacon** approach that will be discussed in this chapter.

Molecular Beacons (MB) are small oligonucleotides that are designed in a stem-loop structure. With a fluorophore attached at one end of the MB and a quencher at the respective other end, the MB remains non-fluorescent, as long as it stays in this stem-loop state. The loop region of the MB is complementary to the RNA or DNA sequence of interest (_target_). Thermodynamically MBs are designed in such a way that the stem-loop structure opens up when binding to its target sequence.\(^{60}\)

![Figure 30: General principle of Molecular Beacons (MB). The MB is designed as stem-loop structure. Like this, a fluorophore (magenta/pink) at one end is efficiently quenched by a quencher (black) at the respective other end. In the presence of its complementary target sequence, the structure is opened up and fluorescence can be observed.\(^{60}\)](image)

Design of Molecular Beacons

The fact that MBs are designed in an entropically favoured intra-molecular stem-loop structure makes the design quite sophisticated. In the absence of target, the MB should stay within the stem-loop structure. Only this structure ensures the close proximity of fluorophore and quencher in contrast to random tangled structures. MBs are only usable within the temperature range that ensures the existence of the stem-loop. In the presence of the target, the target sequence should bind to the closed form of the MB and as a result the stem-loop is forced to open up. This procedure occurs in a three step mechanism. First, the target sequence binds to the closed form of the MB. Further binding will then overcome the energy barrier and open the stem-loop in a time range of seconds to minutes. Once the stem-loop structure is opened, the remaining base pairing occurs in a rather fast time scale.\(^{61}\)

From a thermodynamic point of view, there are two influencing factors: the Gibbs free energy \(\Delta G_S\) for the closed stem-loop structured MB and \(\Delta G_H\) for the formation of the hybrid structure (i.e. MB bound to target sequence). Under certain controlled conditions regarding comparable probe and salt concentrations and solvent, these two factors can also be described by the respective melting temperatures \(T_S\) and \(T_H\) and their ratio, respectively.\(^{61,62}\)
For the design of an MB, it is important that $T_S$ is smaller than $T_H$. Otherwise the binding of the target would not lead to an opening of the stem-loop for thermodynamic reasons. Regarding the specificity of an MB for its target, the two melting temperatures should be close together to allow for single-mismatch discrimination. Even though the loop sequence is much longer (15-25 nucleotides) than the stem (5-7 base pairs), $T_S$ and $T_H$ can still be similar due to the entropically favoured intra-molecular formation of a stem-loop. For this reason, one single mismatch of target sequence and MB can decrease the melting temperature $T_H$ enough to prevent opening of the stem-loop. Tsourkas et al. observed that mismatches in the middle of the loop sequence have the highest impact, most likely because already the first binding step is disturbed.\textsuperscript{61,62}

Interestingly, the two melting temperatures are not fully independent from each other. If $T_S$ is increased due to elongation of the stem sequence or increasing G-C base content, $T_H$ decreases slightly. It can be hypothesised that a target will be more likely to dissociate from the MB even after initial binding, if the alternative conformation is more stable. In addition, the influence of modifications should not be neglected. Some fluorophores (e.g. pyrene) are able to intercalate into the base stacking. Alternatively, fluorescent dyes and quenchers can also stack with each other due to their usually big π-electron system.

Worth mentioning, the entropically favoured stem-loop structure prevents MBs from forming dimeric structures.\textsuperscript{63}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure31.png}
\caption{Melting temperature diagram of a Molecular Beacon (MB) with (blue) and without (red) target sequence. The conformation of the MB is determined via its fluorescence intensity. In the absence of the complementary target sequence, the MB stays in its dark stem-loop state, until the melting temperature of the stem region ($T_S$) is reached, causing fluorescence increase. In the presence of target, the target is separated from the MB, when the melting temperature of the hybrid ($T_H$) is reached. The fluorescence decreases, as the MB regains its dark stem-loop state ($T_H < T_S$), and finally slightly increases again.\textsuperscript{64}}
\end{figure}

A number of design rules for MBs follow from the above. Due the increased entropy of the intra-molecular stem-loop structure, the loop needs to be significantly longer (3-5x) than the stem, which is usually designed with five to seven base pairs. Nevertheless, a loop sequence with more than 30 nucleotides would lead to a decreased specificity and single-nucleotide mismatches could not be separated from perfect complementarity. Additionally, a long loop impairs binding kinetics of the target sequence to the MB, while in contrast a shorter loop sequence might not target a unique sequence within a cell. For this reason it is important to choose a suitable design for the according application. Single-mismatch mutation studies will require another specificity of the MB than RNA tracking experiments.\textsuperscript{63}
Independently from the application, the target sequence must be unique and easily accessible by the MB. This can be tested in advance using the open-access programmes basic local alignment search tool (BLAST) and mfold. Additional secondary structures of the MB itself should be excluded using mfold as well.

A lot of published MBs with good signal-to-noise ratios (SNR) show a G-C content of 40% in the loop sequence and 75% in the stem. Interestingly, there is also an approach called shared stem Molecular Beacon. In this case the target complementary sequence is also part of the stem, so that the stem-loop structure is opened more efficiently and faster, producing more stable hybrids. Furthermore, the fluorophore should not be attached next to a guanosine, as purines, in particular guanosine, are known to quench fluorescence.

Chemical Modifications

To date, a wide range of MB designs have been optimised. Initial issues such as intracellular digestion could be overcome by nuclease-resistant chemical modifications. The usage of 2'-O-methyl-RNA, 2'-fluoro-RNA or the so-called locked nucleic acid (LNA) prevents the MB from digestion, which would ultimately lead to the separation of fluorophore and quencher and thus false positive results. Recently, MARAS et al. published so-called tiny Molecular Beacons, a combination of 2'-O-methyl-RNA and LNA. The loop regions of these tiny chimeric MBs can be as small as 10-15 nucleic acids, while normal MBs are up to 25 nucleic acids. This shorter design allows for more cost-efficient and higher yielding syntheses as well as easier purification. Despite their small target recognition site, there have been promising results that these tiny MBs are specific enough to discriminate RNA sequences even with a single-mismatch compared to the target sequence. Generally, an MB should not be exclusively designed of LNA. Due to the stable binding of LNA to DNA or RNA, the selectivity of the MB would be decreased. Peptide analogues called PNA (abbrev.: peptide nucleic acid, cf. Figure 32) are also nuclease resistant and show very high binding affinities, because there is no repulsion of negatively charged phosphates. It must be noted, that PNA is generally less soluble in aqueous media and might therefore be difficult to handle for some biological applications. In particular PNA MBs, but also MBs in general might show increased SNRs in vivo than it has been observed in vitro. In addition to nuclease degradation, there is also the possibility of unspecific binding to protein, for example in non-polar areas, that cause the beacon to open up and give fluorescent signal. Besides, extremely high affinities, as they are observed for LNA or PNA, will ultimately lead to an impaired specificity.
Molecular Beacons

Figure 32: Schematic view of the base pairing between peptide nucleic acid (PNA) and DNA. The relevant covalent functionalities within the backbone are marked in red. While DNA bears a negatively charged phosphate diester backbone, PNA has common peptide bondings. PNA is known to bind to DNA/RNA with a higher affinity than a complementary DNA/RNA strand would bind. This finding can be explained by the fact that there is no repulsion of two negative charges.

Fluorescence and Quencher Choice

In addition to chemical modifications of the nucleic acids, the fluorophore and quencher choice is very important. To understand the advantages and disadvantages of certain fluorophores and quenchers, the following subchapter will discuss some theoretical background knowledge on fluorescence and quenching types.

Basics in Fluorescence and Quenching Mechanisms

Generally, fluorescence is the ability of a molecule, an atom, or nanostructures to emit a photon, while relaxing to its ground state. Organic dyes are often characterised by a poly-aromatic system or hetero-cycles with an extended π-electron system. The molecule has previously been excited to a higher quantum state by light. After relaxation, the fluorophore is excitable again. This is one of the main causes for the high sensitivity of fluorescence. The fact that the excitation is caused by light is the main difference to chemiluminescence, where the light emission is caused by a chemical reaction. It should be noted that relaxation is also possible via alternative routes. One example is the interaction with another molecule (quenching) and will be discussed later. An additional deactivation process that competes with fluorescence is the intersystem crossing (ISC) to the triplet state. This process can either lead to non-radiative relaxation or even to phosphorescence.
The electric states and the transmission between them are illustrated by the *Jablonski diagram* (depicted in Figure 33).

![Figure 33: Jablonski diagram illustrating the electronic states of a fluorophore and the transitions between these electronic states. The states are arranged in a vertical manner by energy and horizontally by spin multiplicity. Upon light absorption, the dye is excited to a higher energy level. It can either drop to its ground state through fluorescence emission or intersystem crossings (ISC) into triplet states may occur followed by phosphorescence.]

Similar to the photochemistry of photolabile protecting groups, the fluorescence quantum yield determines the effectiveness of a fluorophore. It can either be determined by the ratio of emitted photons to absorbed photons or alternatively, by the fluorescent rate constant $k_f$ to the summarised rates of all excited state decays ($k_i$) including non-radiative mechanisms such as quenching and intersystem crossings.\(^7^3\)

$$\phi = \frac{N(\text{emitted photons})}{N(\text{absorbed photons})}$$

$$\phi = \frac{k_f}{\Sigma_i k_i}$$

The fluorescence lifetime is determined by the average time the system stays in the excited state. The time period is usually in a range of 0.5 to 20 ns.\(^7^3\)

$$[S^*_i] = [S^*_i]_0 * e^{-t\Sigma_i k_i}$$

There are some general rules that underlie fluorescence.

1. **Kasha-Vavilov rule**: even though there are several aberrant cases, even for simple molecules, the rule states that the quantum yield $\phi$ is independent from the excitation wavelength $\lambda_{ex}$.\(^7^5\)
2. **Mirror image rule**: For most fluorescent dyes, the absorption spectra are a mirror image of the fluorescence emission spectra. When the excitation wavelength is altered, the profile of the respective emission spectrum stays unaffected, but the fluorescence intensity correlates to the amplitude of the excitation spectrum.\(^7^3\)
3. **Stokes shift**: The difference of the excitation and emission wavelength with the maximal amplitude in the fluorescence spectrum is referred to as Stokes shift. A big
Stokes shift is usually desired, because in this case the detected emission can be well separated from the excitation light and its scattered light. Like this, a low background is observed that improves the sensitivity of fluorescent techniques. The physical reason for the Stokes shift is the fact that after excitation, the systems drops to the lowest vibrational level of the excited state. Additionally, the emission is often left in the highest level of the ground state.\textsuperscript{73,74}

The loss or suppression of fluorescence can be either desired in the case MBs with the help of a \textit{quencher} or undesired due to unforeseen side reactions or complex formation. One important limiting factor of organic fluorophores is the fact that they ultimately photobleach under long-lasting and high-intensity illumination. Photobleaching is defined as the irreversible loss of fluorescence. The excited fluorophore can be destroyed due to the generation of reactive oxygen species such as singlet oxygen $^1$O$_2$. It is possible to add certain buffer systems, which contain dithiols, during imaging applications to prevent oxidation or reduce oxidised dyes.\textsuperscript{76,77}

Regarding fluorescence quenching, it can be differentiated between \textit{dynamic} and \textit{static quenching}.

\textit{Static Quenching}

Also known as \textit{contact quenching} or \textit{ground-state complex formation}, static quenching means that the quenching occurs in the ground-state of the fluorophore prior to excitation. Mostly due to hydrophobic effects, organic dyes can stack onto each other within aqueous media, forming non-fluorescent dimeric complexes. This dimer shows an altered, characteristic absorption spectrum, which is unique for the newly formed fluorophore-quencher complex. After absorption of energy, the energetically excited complex immediately returns to its ground state without emission of light. Due to the requirement of complex formation, this form of quenching can only occur, if fluorophore and quencher are adjacent to each other with a distance below 20 Å. Additionally, this form of quenching is highly temperature dependent and fluorophore-quencher complexes can be disrupted by surfactants.\textsuperscript{73}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{static_quenching.png}
\caption{Mechanism of static quenching. Due to hydrophobic interactions, two organic molecules (R / Q) can form a non-fluorescent ground-state complex (RQ).}
\end{figure}
Dynamic Quenching

This form of quenching means that the deactivation of the fluorescence occurs, while the fluorophore is in the excited state. One can differentiate between Collision Quenching (Dexter Quenching) and Förster Resonance Energy Transfer (FRET).

Regarding Collisional quenching, the fluorophore is deactivated upon short-range contact with another molecule (<10 Å). Examples for collision quenchers are oxygen, organic amines, and halides such as iodide. One well-known application is the visualisation of DNA by ethidium bromide. If ethidium bromide is intercalated within the DNA base stacking, it is protected from being collision quenched by oxygen. In contrast to static quenching, the spectrum of the fluorophore stays unchanged. The quenching is based on bilateral electron exchange between fluorophore and quencher. Worth noting, the fluorophore can be excited either in its singlet or in its triplet state (cf. Figure 35). Collision quenching in solution is proportional to temperature, as a higher temperature leads to increased diffusion rates.  

![Figure 35: In the case of Dexter energy transfer, two molecules bilaterally exchange their electrons. The reaction rate constant of Dexter energy transfer exponentially decays with increasing distance of the two molecules.](image)

In contrast, FRET requires a spectral overlap of the emission spectrum of the fluorophore (donor) with the absorption (excitation) spectrum of the quencher (acceptor). The energy of the excited donor dye is non-radiatively transferred to the donor in its ground state. The acceptor dye can either relax by emission of fluorescence (fluorescent quencher) or heat (dark quencher). FRET is based on dipole-dipole interactions between donor and acceptor. Therefore, the quenching efficiency is dependent on dipole-orientation. In addition to spectral overlap and dipole orientation, the distance should be in a range of 10-100 Å. Generally, quenching efficiency decreases following $1/R^6$ with $R$ as the distance between fluorophore and quencher. Worth noting, the FRET effect is not dependent on temperature except for certain DNA-based applications, if the melting of a DNA-duplex causes separation of donor and acceptor.
Molecular Beacons

Figure 36: Mechanism of FRET quenching. If a spectral overlap of the emission spectrum of the fluorophore with the absorption spectrum of the quencher exists, the energy of the excited donor dye can be transferred non-radiatively to the donor in its ground state.

As mentioned in previous subchapters, fluorescent dyes can be incorporated during solid-phase synthesis, if they are available as phosphoramidite and if they survive all steps of oligonucleotide synthesis, deprotection, and purification. Commercially available fluorophore phosphoramidites include Fluorescein (FAM) and some of its derivatives (hexachloro-Fluorescein HEX, tetrachloro-Fluorescein TET), tetramethyl-rhodamin (TAMRA), and the cyanine dye Cy3. While these dyes are easy to incorporate into an oligonucleotide without additional purification steps, modern dyes such as Atto and AlexaFluor dyes are superior in their photochemical characteristics. To covalently bind dyes that are not available as phosphoramidite to an oligonucleotide, chemical functionalities must be introduced into the oligonucleotide instead. These modifications and the according labelling procedure has been introduced and discussed above.

When choosing the position of the dye, it must be considered that some environmental factors such as the base that is adjacent to the dye might affect its quantum yield. The quenching effect of a neighbouring guanosine is well-known and can be utilised for the design of quencher-free MBs (see below). Most likely, the underlying mechanism of this fluorophore quenching is electron migration, depending on the fluorophore, the reduction of quantum yield can be up to 40%.

Quantum Dots

As an alternative to the usage or organic fluorescent dyes, there are also nanostructured inorganic semiconductor materials available that are fluorescent with superior photochemical characteristics – so-called quantum dots (QD). These nanoparticles are characterised by their high photostability, sharp emission peaks and extensive Stokes shift. Due to their small size (2-10 nm) and the high surface-to-volume ratio, QDs exhibit quantum mechanical properties. This phenomenon is referred to as quantum confinement effect. This is the case, if the size of the nanoparticle is smaller than the exciton Bohr radius, which is the distance of the hole in the valence band to the electron position in the conductance band. While the excitation spectrum is the same for the same type of QDs, the emission spectrum can be altered by the size of the QD or additional modifications of the shell. While core-type QDs (e.g. CdS / CdSe) can only be modified by their size, core-shell-type QDs, which are enclosed by a shell of a higher band gap semiconductor (e.g. ZnS), can also be modified by exchange of the shell material.
Figure 37: Energy diagram of a usual bulky semiconductor (left) compared to quantum dots with differing size (right). The colour indicates the “fluorescence colour”. When the size of the semiconductor nanocrystal is smaller than the exciton Bohr radius, quantisation of the energy levels occurs. As a consequence, the behaviour of QDs is more closely related to atoms than to bulk materials. Generally, with decreasing size of the crystal, the difference in energy between the highest valence band and the lowest conduction band increases.81,82

Another advantage of QDs becomes obvious, when applying QDs on Molecular Beacons. Usual MBs that have been labelled with organic fluorophores can be found within the nucleus after a certain time frame within a cell. Due to the increased size of quantum dots, this nuclear background fluorescence is not observed in in vivo applications.83,84

Special Designs of Molecular Beacons
With this knowledge on fluorescence, some special MB designs will be introduced. Several designs have been published that use a Molecular Beacon with two Pyrene dyes in the 3’- and 5’-stem region. The two intercalating dyes are able to form a so-called excited dimer (excimer). An excimer is characterised by one of the two molecules being in the excited state, for example due to excitation by light. When the excited molecule within the excimer decays to the ground state, the emission wavelength of the fluorescent excimer is longer (energetically lower) than that of the excited monomer.85–87

Figure 38: Principle mechanism of an excimer-based MB. Due to complex formation of two Pyrene dyes, the fluorescence changes upon target binding of the MB. As a consequence, the two monomeric Pyrenes show an altered fluorescence spectrum.80
In 2000, Tyagi and Kramer further developed their initial MB design and used two fluorescent dyes that form a FRET pair called wavelength-shifting MB. In the absence of target, the MB stays dark, because the fluorescence is quenched as for common MB designs. In the presence of the target and upon opening of the stem-loop structure the so-called harvester fluorophore is separated from the dark quencher and causes fluorescence emission of the emitter fluorophore by FRET (cf. Figure 39). The authors claim a brighter signal that is further away from the excitation wavelength compared to a single fluorophore, causing a lower SNR.

In 2004, Tsourkas et al. published so-called dual-FRET MBs. In this case two MBs need to bind to the target sequence directly next to each other with neighbouring fluorophores, which are chosen as a FRET pair (cf. Figure 40). With this design, even nuclease digestion or unspecific binding to similar target sequences would not cause false positive results. Therefore, the Molecular Beacons must be designed as shared stem MBs that contain target complementary sequence parts within the stem structure to ensure direct contact between the fluorophores.
Similar to the above mentioned approaches, an MB can bear several dark quenchers, so called superquenching MBs. By using three quenchers per MB, WEIHONG TAN et al. could improve the SNR more than 100-fold compared to a commonly discussed single-quencher MB.\textsuperscript{93}

**Applications for MBs**

Molecular Beacons have been applied in a number of different research areas, among them biochemistry, medical diagnostics, or material sciences. Already TYAGI AND KRAMER used their invention in 1996 for quantitative real-time PCR (qPCR, abbrev.: quantitative polymerase chain reaction).\textsuperscript{60} The sequence that needs to be amplified bears the target sequence for the Molecular Beacon. During the annealing step of the PCR, not only primers may bind to the target, but also the MB causing a fluorescent readout. This approach is much more sensitive and specific regarding the presence of unknown target sequences within a mixture of different sequences compared to other fluorescent qPCR markers, e.g SYBR\textsuperscript{©} dyes. Therefore MBs have already been applied in diagnostic qPCRs to detect and analyse viral infections.\textsuperscript{94,95} Like all oligonucleotides, MBs can be immobilised on surfaces to produce biosensor microchips with a quantitative fluorescent readout. For this purpose, MBs have been immobilised on graphene or gold surfaces for analytical detection of certain target sequences.\textsuperscript{96} Especially when using gold, no additional quencher is necessary, as gold has been shown to quench fluorescence producing a good SNR.\textsuperscript{97} Recently, LIBERA et al. used biotinylated PEG microgels to immobilise streptavidin-bearing MBs.\textsuperscript{98} In contrast to solid surfaces, the group could show that the SNR can be massively improved, most likely due to the lack of common non-specific interactions of MBs with common solid surfaces. It can be hypothesised that in particular apolar organic dyes may interact with the surface and, by changing the conformation, increasing the background signal of an MB.\textsuperscript{98} The general advantage of the use of MBs on analytical biosensor chips is the fact that no additional washing step is necessary, because the MB quantitatively reflects the amount of the target sequence.

![Figure 41: Schematic view of immobilised Molecular Beacons (MBs). In this case, MBs are immobilised on biotinylated PEG hydrogels. The MBs stay in their closed conformation (A), until they are exposed to their target sequence (B). In contrast to solid surfaces, the usage of hydrogels prevents unspecific interactions of the organic dyes with the surface. As a result, the signal-to-noise ratio is massively improved. No signal is detectable in the absence of the complementary target sequence (cf. C before and D after addition of target).\textsuperscript{98}](image-url)
**Alternative Methods**

Even though MBs are of special interest for the detection of their complementary target sequences, a number of studies prove their value for the detection of DNA/RNA-protein interactions. Common analysis methods include ELISA analysis, polyacrylamide gels, and Western blotting experiments that are rather time consuming. Recombinant proteins can also be expressed that are fused to fluorescent proteins. This can be a drawback, when native mRNA transport or distribution is supposed to be studied, that might be altered by the presence of the fusion protein. MBs are of special interest for single strand binding proteins, as it has been shown by Tan et al.

One biomedically relevant example has been published in 2001 by Yamamoto et al. for the HIV-relevant Tat protein (trans-activator of transcription). The Tat protein is a viral transcription factor that enhances transcription efficiency significantly. Certain RNA sequences bind tightly to the Tat protein. Yamamoto et al. used certain parts of these known sequences for their MB loop sequence. Upon binding by Tat, a conformational change causes a fluorescent readout within several minutes.

In 2012 Wei Hong Tan and his group published an anti-His-tag MB (AHMB) that opens its stem-loop structure when binding to His-tagged proteins. Like this, the concentration of recombinant His-tagged proteins can be determined in a very time-efficient way.

One major application of MBs is the analysis of RNA distribution within a cell that is still heavily discussed in literature. Active transport of messengerRNA (mRNA), for instance, is thought to be a key process in locally controlled protein synthesis. Several methods have been developed to further elucidate RNA transport and distribution within a cell. Among others, the so-called MS2-GFP system has been used to visualise mRNA in a living cell. In this technique, an MS2-GFP fusion protein recognises stem-loop structural multimers that have been coexpressed into the 3’-untranslated region (UTR) of the reporter RNA (cf. Figure 42). One advantage of this technique is the fact that cells do not need to be fixed, so that in vivo RNA analysis becomes possible.

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**Figure 42:** The MS2-GFP technique is based on the natural interaction of the MS2 coat protein (MS2 CP) with certain stem-loop structures within an RNA. Stem-loop-structure containing mRNAs can be visualised and its appearance can be monitored within living cells, if the MS2 CP is in complex with GFP.
Other techniques such as *Fluorescence In Situ Hybridisation* (FISH) use fluorescently labelled oligonucleotides that are complementary to the RNA of interest. Both techniques have major drawbacks. The MS2 approach, for instance, cannot visualise native RNA, but cells need to be transfected with both the RNA stem-loop construct and the MS2-GFP fusion protein. In contrast, FISH is able to bind to native RNA and DNA, but it cannot be distinguished between bound and unbound state.

One additional technique that is worth mentioning is the so-called *Spinach RNA* approach that has been published by JAFFREY et al. in 2011. The group uses RNA aptamers that can bind organic fluorescent dyes, e.g. DFHBI (3,5-difluoro-4-hydroxybenzylidene imidazolinone). Exclusively in the bound state, the dye is able to fluoresce by cis-trans isomerisation. The group could successfully visualise modified RNA, carrying the dye-aptamer site, as well as proteins by using RNA aptamers with an additional protein binding site. Even a variety of different fluorescence spectra became available by changing substituents of the organic dye. As for the MS2-GFP approach, the RNA of interest needs to be derivatised to contain the dye-binding aptamer region. Consequently this technique does not allow for the visualisation of native RNA.

Most of the previously mentioned disadvantages can be overcome by using MBs to visualise RNA *in vivo*. In 2003, BRATU et al. analysed the movement of native mRNAs in Drosophila oocytes and the influence of cytoskeleton rearrangement of mRNA transport using MBs. Using MBs, the native localisation and developmental concentration change of RNAs can be visualised in living cells. It is even possible to detect several mRNAs within a cell by using differently fluorescent MBs.
Synaptic Plasticity

Synaptic Plasticity in Neuronal Hippocampal Cells

The process of memory and learning is based on a network of neuronal cells and changes of the strength in their connection. The underlying processes of this cellular adaption are highly complex and dependent on various regulatory processes. These processes are generally referred to as synaptic plasticity.

The following subchapters discuss the morphology and additionally, some general processes of neuronal cells will be explained. Following that, the molecular process of memory and learning as well as its regulatory mechanisms will be elucidated.

The Hippocampus

The hippocampus is part of the brain and is named in Greek due to its morphological resemblance to a sea horse. In the mammal brain, there is one hippocampus in each hemisphere that can be found in the medial temporal lobe of humans. It has been shown that one hippocampus is sufficient to undertake the hippocampal tasks. Among other hypothesised tasks, it is widely accepted that the hippocampus has an important role in short-term memory as well as spatial orientation. Especially the medical case of Henry Molaison (sometimes referred to as “H.M.” in literature) elucidated the hippocampal role in memory formation. In 1953, Molaison's medial temporal lobe including the hippocampus was resected. Despite the success in curing Molaison’s epilepsy, the patient was left without the ability to form new memories. Even though he suffered from complete anterograde amnesia, he was still able to remember situations that happened prior to the surgery. Besides, some forms of memory seem to be independent from the hippocampus, since patients with anterograde amnesia are able to learn new skills such as playing an instrument. Also widely debated is its task in spatial orientation. After resection, rats are not able to orientate in their environment.

Interestingly, the hippocampus is one of the first parts that is destroyed during Alzheimer's disease. Therefore, the hippocampus is one of the most intensively studied parts regarding the mechanisms of memory and learning.

Anatomically, the hippocampus is formed like a sea horse-shaped curved tube that can be divided in its two main parts, the Ammon's horn and the dentate gyrus (DG). The DG lies midmost and is a separate structure consisting of granule cells. Starting there, four cornu ammonis (CA4 to CA1) areas turning to the outer part with the outmost being the biggest one. The name of this area derives from the Egyptian God Ammon and its similarity to a goat’s horn. The CA parts consist of pyramidal cells that are characteristic for the Hippocampus. The name is based on the fact that these cells have a triangular soma. Following CA1, the subiculum can be found. This area of the hippocampus is thought to play the major role in epilepsy and Alzheimer's disease, because amyloid-β-plaques are first detected in this area. Adjoining and slightly below the subiculum, the entorhinal cortex (EC) is located. The EC does not belong to the hippocampus, but is directly connected to it due to the pathway of signal conduction.
Most incoming signals within the hippocampus start in certain parts of the EC. As shown in Figure 44, the signal is conducted to DG and CA3, finally terminating at CA1. In addition there is a direct pathway connecting EC and CA1. Neurons of the CA1 area lead to the subiculum and other areas of the EC. Like that, a loop-like signal conduction results. The conduction pathway starting at CA3 and projecting to CA1 is called Schaffer collateral and is thought to be an important area of memory formation. For this reason, the neurons of this part are heavily studied to understand the adaption of the neuronal network during memory formation also known as synaptic plasticity. In addition, this area is a potential drug target for dementia treatment.

The Morphology of Neuronal Cells

The nervous system consists of highly complex polar cells that basically consist of antennae for incoming signals, a cell body for information collection and processing, and a stimulus forwarding part. These stimuli can either be chemical or even electrical, because neurons are electrically excitable cells.

Even though there is a wide diversity within neuronal morphology, most neurons share some anatomical mutualities, as it is schematically shown in Figure 45. The cell body (soma) is usually the biggest and most compact part of the cell. Like in other cells, it includes most of the organelles such as nucleus, endoplasmatic reticulum (ER), and mitochondria.

The information antennae for incoming signals are called dendrites that extrude from the soma. There are usually several dendrites per neuron and each of them branches several times. The further away from the soma the thinner dendrites usually become. Incoming signals coming from the dendrites are collected and further processed by the cell, as it is described later in the introduction.

In contrast, outgoing electrical signals are summated and generated in the axon hillock that connects the soma with the cell’s axon. Signals to other neurons or cells are then transmitted electrochemically by the axon. It is important to note that there is only one axon independently from the neuron type, while there are numerous dendrites.
Depending on the organism, the axon is electrically insulated by the Myelin sheath. The myelation consists of certain glia cell types. While the myelination of the central nervous system (CNS) is built up by so-called oligodendrocytes, it is formed by Schwann cells in the peripheral neurons. Between two myelin sheaths there is an uninsulated constriction called Ranvier node. This insulation of the axon accelerates the electrical conduction in vertebrates (salutary conduction).\(^{124}\)

At their distal part, axons can branch into a number of telodendria (from ancient greek: “tree” and “end”). Finally each telodendron branches into axon terminals (cf. subchapter “Synapse Function” below). There, the electrical information is translated into a chemical one by triggering the secretion of neurotransmitters. These are stored within vesicles. The axon terminal is in close proximity to the adjacent cell’s dendrites.\(^{124}\)

The process of signal conduction is explained in the following subchapters in more detail.

**Stimuli Conduction in Neuronal Cells**

Under physiological conditions, ions are not distributed equally in- and outside of a cell causing an electrical tension at the membrane. The resulting resting potential of a cell is dependent on the cell type and is measured as -60 to -70 mV for most cells including neuronal cells. The negative value means that the intracellular area is less positive. This phenomenon is often explained by the presence of a Na\(^+\)/K\(^+\) pump that leads to a concentration difference of these two ions. In an ATP-dependent manner, three Na\(^+\) ions are exported, while two K\(^+\) ions are transported into the cell. Regarding the resting potential in a more detailed manner, it becomes obvious that the Na\(^+\)/K\(^+\) pump cannot fully explain the value. The value differs mainly from the expected Nernst values of the ions due to leakage currents of mostly Na\(^+\), but also Ca\(^{2+}\) and Cl\(^-\) ions. Additionally it must be noted that intracellular and possibly charged proteins are immobile and not able to perform charge equilibration at all.\(^{124}\)

In summary, several reasons can be named why the resting potential massively differs from the theoretically calculated Nernst potential:
• the different equilibrium potentials of respective ions
• their different membrane permeability
• the presence of ion channels that allow only certain ions to pass through
• immobile charged components of a cell
• ion binding components within a cell

In neuronal cells, a change of the resting potential is responsible for signal triggering and transmission. Incoming signals trigger the opening of ion channels, as it is explained in more detail below. When neurotransmitters, which have been secreted from the pre-synaptic neuron, open ligand-gated ion channels at the membrane of the post-synaptic neuron, the increased ion flux changes the membrane potential locally in the dendrites.

If the potential is increased due to cation influx (mostly Na\(^+\) and Ca\(^{2+}\)), it is called *excitatory post-synaptic potential* (EPSP). The ion flow causing the EPSP is sometimes referred to as *excitatory post-synaptic current* (EPSC). In vertebrates, the most important neurotransmitter that is responsible for EPSPs is the amino acid glutamate. Regarding the neuromuscular action as well as invertebrates, acetylcholine should also be mentioned as EPSP causing.

In contrast to EPSPs, which depolarise the membrane, there is the inhibitory post-synaptic potential (IPSP). The most important neurotransmitters involved in IPSPs are glycine and \(\gamma\)-amino butyric acid (GABA). An IPSP can be caused due to either K\(^+\) efflux or Cl\(^-\) influx, both causing a hyperpolarisation of the cell. Hyperpolarisation means that the membrane potential becomes more negative compared to the resting potential.

EPSPs and IPSPs can be graded locally and temporally. The sum of all incoming post-synaptic potentials (PSPs) is collected at the axon hillock. When the sum of all incoming PSPs overcomes a certain threshold, the neuron can fire a so-called *action potential* (AP) along the axon (cf. Figure 46).

Within large neurons, the PSP is more likely to partly lose its strength on its way from distal dendrites to the soma. Additionally, multiple PSPs that occur during a time frame of approx. 1-2 ms can sum up and by that overcome the threshold for an action potential. EPSPs and IPSPs can also compete with each other. While EPSPs increase the likelihood for an AP to happen, IPSPs reduce it.

If the sum of the PSPs at the axon hillock exceeds a threshold of -50 mV, an AP can occur. The threshold value of -50 mV means that a depolarisation of 20 mV compared to the resting potential of -70 mV is necessary. Looking at an AP more closely, it must be mentioned that the course of an AP is always identical. In contrast to PSPs, the AP does not have an additive effect and consequently a sub-threshold depolarisation does not cause an AP.

Following the depolarisation, which exceeds the threshold, voltage-gated sodium (Na\(_v\)) channels, which are located along the axon, open up. Due to the unequal Na\(^+\) ion distribution, the electrostatic pressure causes Na\(^+\) ion influx. Simply spoken, this Na\(^+\) influx causes a further depolarisation and following that the opening of additional Na\(^+\) channels. So, the sudden sodium ion influx within 1 ms overshoots the membrane potential to +50 mV and is based on a positive feedback mechanism.

After approx. 5 ms, the Na\(_v\) channels come back to an inactive state that is also closed, but cannot be reactivated for a couple of milliseconds. On a molecular level, highly charged transmembrane helices turn outward in response to the depolarisation allowing sodium ions to pass through the *activation gate* (often referred to as “A gate” in literature). An
additional inactivation gate (I gate) consists of a cytoplasmatic domain that becomes available shortly after the initial conformation change and binds to a docking site. This inactivation domain – or "particle" – is able to inactivate the Na\textsubscript{v} channel and makes them unresponsive for an additional activation.\textsuperscript{127}

While Na\textsubscript{v} channels can change their conformation from a deactivated closed state to the active open state within 1 ms and finally to the inactive ("blocked") closed state, voltage-gated K\textsuperscript{+} (K\textsubscript{v}) channels need up to 10 ms for the comparable conformational change to the open state. It is important to note that the term "open" or "closed" should not be understood as an absolute state in the context of ion channels. It rather means the increased or decreased probability for ions to be able to pass through.

Following the time-delayed opening of the K\textsubscript{v} channels, K\textsuperscript{+} ions are allowed to efflux according to their electrochemical gradient. Due to K\textsuperscript{+} ion efflux, the membrane potential repolarises and even hyperpolarises below the resting potential to approx. -90 mV. This hyperpolarisation occurs due to temporally increased potassium ion conductivity. One reason for hyperpolarisation is the rather slow conformational change of K\textsubscript{v} channels in general. In addition, not only Na\textsuperscript{+} ions, but even a small amount of Ca\textsuperscript{2+} ions are able to enter the cell through voltage-gated Calcium ion (Ca\textsubscript{v}) channels during the initial depolarisation phase. The slightly increased concentration of Ca\textsuperscript{2+} ions within the cells opens additional Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels, so that the K\textsuperscript{+} efflux is further enhanced and hyperpolarisation is more likely to happen. Furthermore, special inward-rectifying K\textsuperscript{+} ion (K\textsubscript{ir}) channels, which are usually supposed to stabilise the resting potential by passing K\textsuperscript{+} ions into the cell, are blocked by small, mostly positively charged molecules such as the polyamine spermine during phases of depolarisation. These K\textsubscript{ir} channels only reopen during hyperpolarisation in order to restabilise the resting potential.\textsuperscript{128-130}

![Figure 46: The figure shows a plot of a typical action potential with its different phases. The membrane potential starts at -60 to -70 mV. If a stimulus raises the membrane potential above -55 mV (the threshold potential), the membrane potential rapidly rises to a peak potential of +40 mV within 1 ms. The potential then drops and overshoots down to -90 mV, and finally the resting potential of -70 mV is reestablished within 3 to 4 ms.\textsuperscript{131}](image)

Directly after an action potential, neurons are not able respond to stimuli again for a certain time frame that is referred to as refractory period. The refractory period can be divided into the absolute and relative one. The absolute refractory period is characterised by Na\textsubscript{v} channels in their inactive closed state. The neuron cannot be activated during this time
independently of the strength of incoming stimuli. As already mentioned above, the reason for channel blockage is caused by an intrinsic blocking particle.\textsuperscript{132} In contrast to the absolute refractory period, during the relative phase the cell can be activated by comparatively strong stimuli. The relative refractory period is caused by an enhanced number of K\textsuperscript{+} ion channels that are still more likely to be opened than usually. Higher K\textsuperscript{+} ion efflux makes it difficult for the cell to depolarise above the threshold necessary for AP firing, even though most Na\textsubscript{v} channels have returned to their activatable state.

Importantly, the refractory period is also responsible for the AP to move exclusively unidirectionally along the axon to the synapses, as Na\textsubscript{v} channels that are closer to the soma are not activatable yet.\textsuperscript{132} It must be mentioned that the AP would travel along the axon with approx. 1-30 m/s in the way it is explained above. Especially in vertebrates with their long and thin neurons (up to 1 m length and 0.05-20 µm in diameter), the AP amplitude would slowly decrease, before it reaches the synapse. For this reason the vertebrate axon is insulated by lipid-rich Myelin sheath. As mentioned in a previous subchapter, this sheath is made of certain glia cells, the Schwan cells.\textsuperscript{124,133}

As a result, the AP is precluded from occurring at insulated areas and is solely possible at areas along the axon where the insulation is separated – the Ranvier nodes. This causes the AP to skip from one node of Ranvier to the next. This type of AP conduction along vertebrate axons is called saltatory conduction and, compared to unmyelinated axons, the conduction is accelerated to up to 200 m/s. Moreover the AP is stable in its amplitude over a wide range.\textsuperscript{124,133}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure47.png}
\caption{In vertebrates, the signal migration is accelerated by the presence of myelin sheath. This insulation prevents the action potential from happening along the whole axon (A), but only at Ranvier nodes (B, “active area”). Like this, the AP jumps from one node to the next. This form of signal transduction is called saltatory conduction (B).\textsuperscript{134}}
\end{figure}

Once the AP has finally reached the synapses, voltage-gated Ca\textsuperscript{2+} channels are opened. Following the Ca\textsuperscript{2+} influx, transmitter filled vesicles are secreted into the synaptic cleft. The transmitter molecules diffuse to the post-synaptic membrane where they bind to ligand-gated ion channels and consequently cause PSPs within the post-synaptic neuron.\textsuperscript{124} A more detailed view on the synapse function can be found in the next subchapter. In addition, the detailed activity of ligand-gated ion channels is explained later in this chapter focusing on their role in memory formation.
**Synapse Function**

At synapses the electrical signal of the action potential (AP), that has travelled along the axon, is translated into a chemical one by the release of small organic molecules named neurotransmitters.\(^{124}\)

When the action potential reaches the axon terminal, the plasma membrane is depolarised. Voltage-gated calcium ion channels open up and, with the outer Ca\(^{2+}\) concentration being 1000 times higher, a Ca\(^{2+}\) influx is induced. The increased Ca\(^{2+}\) concentration triggers the exocytosis of vesicles. The vesicles are filled with up to 10,000 neurotransmitter molecules and their size differs between 50 nm and 200 nm.\(^{124}\)

Within the axon terminal, newly synthesised or recycled neurotransmitter vesicles are located further away from the pre-synaptic membrane in a storage or reserve pool. These reserve vesicles can be transported actively closer to the pre-synaptic membrane. This area is called *active area* and contains the readily releasable pool of neurotransmitter filled vesicles.\(^ {135}\)

The reserve pool of vesicles is hold in place by e.g. Synapsin I and II that are bound to both the vesicles and actin. After Ca\(^{2+}\) influx, calmodulin (CaM), a highly conserved regulatory protein, binds to Ca\(^{2+}\). CaM contains four typical loop-helix-loop motifs, the so-called EF-Hands, for calcium binding. Following ion binding, the Ca\(^{2+}/\)calmodulin-dependent kinase II (CaMKII) is activated and, among others, phosphorylates Synapsin.\(^{136-138}\) After phosphorylation, the neurotransmitter vesicles are released from the actin skeleton and are ready for transport to the active zone. The GTP-binding proteins Rab3a and Rab3b, which can also bind to the vesicle membrane, guide the vesicles to the active zone.\(^ {137,138}\)

Once the vesicle has reached the pre-synaptic membrane, a group of proteins called synaptotagmines, which are anchored to the membrane, controls exocytosis depending on the Ca\(^{2+}\) binding. Only in the presence of Ca\(^{2+}\), the vesicle can bind to the membrane and also its fusion to the membrane and the formation of the fusion pore is enabled. Without Ca\(^{2+}\) bound to synaptotagmin, the vesicle stays in a ready-for-fusion state, but no fusion pore can be formed. The formation of a so-called *SNARE-complex* (soluble N-ethylmaleimide-sensitive-factor attachment receptor) assures proper fusion of the vesicle to the membrane.\(^ {139,140}\) In 2006, Frolov et al. could show that Ca\(^{2+}\) binding synaptotagmin is necessary to remove and replace tightly bound blocking proteins such as Complexin from the SNARE complex.\(^ {141}\)

While these are the most important proteins to understand Ca\(^{2+}\)-dependent neurotransmitter release, there are a lot more assisting proteins of this highly complex process.

Once the neurotransmitters have been released in the synaptic cleft, they diffuse to the membrane of the post-synaptic cell. In the dendritic membrane of the post-synaptic cell, ligand-gated ion channels are located to which the diffusing neurotransmitters bind.\(^ {124,131}\)
The Glutamate Receptor Ion Channels

Glutamate receptors are ligand-gated synaptic receptors that channel ions upon L-glutamate binding. The amino acid glutamate is ubiquitous, but especially regarding the CNS, it is responsible for most excitatory signals. This excitatory neurotransmitter can be found in more than 50% of the brain tissue, mostly present in glia cells and neurons. In addition to glutamate-gated ion channels, which are referred to as ionotropic glutamate receptors (iGluRs), metabotropic glutamate receptors (mGluRs) are known. mGluRs are mostly responsible for post-synaptic protein modulation by indirectly activating Ca\(^{2+}\) or K\(^+\) channels via diverse signal cascades. Additionally, they have also been shown to inhibit adenyl cyclase and consequently decrease the concentration of the second messenger cAMP. In contrast to iGluRs, mGluRs are mostly located further away from synapses (peri-synaptic sites). Most likely, mGluRs increase the intracellular Ca\(^{2+}\) concentration from the endoplasmatic reticulum (ER) as a reservoir and hence start Ca\(^{2+}\)-dependent signal cascades. Even though some studies have shown mGluRs to be involved in synaptic plasticity, their detailed role and their interplay with iGluRs in memory formation is still widely unknown and needs further research. For this reason, this and the following subchapter will mainly focus on iGluRs.

Generally, iGluRs are non-selective ion channels that flux Na\(^+\), K\(^+\), and under certain conditions also Ca\(^{2+}\) ions. Consequently, they cause an excitatory response of the post-synaptic cell by depolarising the membrane, as it is explained in the previous chapter.

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**Figure 48:** Generally, iGluRs are formed as tetramers (more specific dimers of pre-formed dimers). Monomers associate strongly through interactions between their N-terminal domains (NTDs). Each subunit is built up in a similar manner: The N-terminal domain forms the ligand (e.g. glutamate) binding domain together with a second extracellular loop. Each subunit can exist in two splice variants (flip and flop) with altered ligand binding sensitivities. After transcription, the mRNA might be edited, resulting in slightly different primary amino acid sequences and for some iGluRs even in different ion transduction specificities.

All iGluRs share some common basic structures. As shown in Figure 48, iGluRs are built up as tetramers. Each monomeric subunit consists of four hydrophobic transmembrane helices (TM I-IV). TM II does not fully span the membrane, and for this reason, this TM domain is often referred to as re-entrant loop in literature. As a consequence, the N-terminal end of each subunit lies extracellularly, while the C-terminus can be found intracellularly. The loop region that connects TM III and TM IV is rather long and forms the binding site for ligands.
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together with parts of the N-terminal end. The C-terminal site is the site of extensive splicing, so that each subunit of the iGluRs exists in diverse isoforms.\textsuperscript{146,147} iGluRs are distinguished according to their different pharmacologies and their agonists, which might even bind stronger than glutamate (cf. Table 1).\textsuperscript{148}

**Table 1: Overview of different glutamate receptors in the central nervous system.** NMDA, AMPA and kainite receptors are ionotropic receptors and are Na\textsuperscript{+} and partially Ca\textsuperscript{2+} permeable upon ligand binding. In contrast, the activation of metabotropic receptors initiates different signal transduction pathways. Apart from glutamate, several other ligands can bind to the receptors with varying affinities. Their structures are shown below.\textsuperscript{148}

<table>
<thead>
<tr>
<th>Subunits</th>
<th>Transduction system</th>
<th>Agonist potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA receptor</td>
<td>GluN1, N2A-D, N3A</td>
<td>Na\textsuperscript{+}, Ca\textsuperscript{2+}</td>
</tr>
<tr>
<td>AMPA receptor</td>
<td>GluA1-4, (5-7)</td>
<td>Na\textsuperscript{+}, K\textsuperscript{+}, (Ca\textsuperscript{2+})</td>
</tr>
<tr>
<td>Kainate receptor</td>
<td>GluK1-5</td>
<td>Na\textsuperscript{+}, Ca\textsuperscript{2+}</td>
</tr>
</tbody>
</table>

**Metabotropic**

- **Type I:** mGluR1,5 - PLC
- **Type II:** mGluR2,3 - adenylyl cyclase inhibition, PLC activation
- **Type III:** mGluR4,6,7,8 - adenylyl cyclase

QA / Glu

![Figure 49: Molecular structures of different glutamate receptor ligands. Each molecule can bind to different types of glutamate receptors with varying affinities. From left to right: glutamate, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainite, quisqualic acid (QA).\textsuperscript{149}](image)

AMPA (abbreviation for: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors are responsible for a fast excitatory synaptic transmission and are built up of subunits GluA1-4 that form tetramers and can either be homo- or heteromeric. Upon glutamate binding, AMPA receptors (AMPARs) mostly channel Na\textsuperscript{+} and Ca\textsuperscript{2+}, causing depolarisation of the post-synaptic membrane.

The N-terminal ending of all subunits exists in two splice variants, which are named *flip* and *flop*. Depending on the splice variant, studies have observed altered desensitisation kinetics. In particular the flop variant is known to be less responsive to AMPAR potentiators and desensitises more rapidly in response to glutamate.\textsuperscript{150}

In literature, the GluA2 subunit is regarded as the most important one, as this subunit determines Ca\textsuperscript{2+} permeability, and its distribution is altered heavily during learning processes. Interestingly, the GluA2 subunit bears a so-called *Q/R-editing* site at the C-terminal side of TM II. This means that in the GluA2 mRNA, *A-to-I* editing takes place by ADAR – a double-stranded RNA specific adenosine deaminase.\textsuperscript{150} This form of RNA editing is
the most common post-transcriptional modification in mammals and occurs in areas of double-stranded RNA structures. By changing adenosine to inosine, which mostly acts as guanosine regarding both RNA secondary structure formation and translation, the amino acid sequence can be changed. In this case, the neutral amino acid glutamine (Q) is substituted by the positively charged arginine (R). The presence of a positive charge in the R-containing GluA2 (GluA2(R)) subunit prevents Ca^{2+} ions from passing through the channel. In contrast, unedited GluA2(Q) containing channels as well as GluA2 lacking ones are able to shuttle Ca^{2+}. The majority of neurons within the CNS has GluA2(R) containing AMPA receptors, and consequently AMPARs are widely regarded as Ca^{2+}-impermeable. Interestingly, both the GluA2 content and the extent of Q/R editing are different depending on the developmental stage and during phases of memory formation. Worth mentioning during early brain development, GluA2 can hardly be found. Instead, GluA1-GluA4 heterotetramers are mostly present, that are later substituted by GluA1-GluA2-heterotetramers in the mature brain. Particularly, GluA2(R) is not able to traffic from the ER to the membrane, as its amino acid sequence contains an ER-retention signal. GluA2 can solely leave the ER as GluA2(Q) homomers or within a GluA1-GluA2(R) complex, which masks the retention sequence.\textsuperscript{150,151}

Another important domain of the GluA2 subunit is its C-terminal ending, which bears a PDZ domain and acts as binding site for a number of interaction proteins. A PDZ domain is a highly conserved modular part within a protein and is important for interactions with and binding to other proteins. Regarding AMPARs, this interaction domain is essential during learning processes, when either AMPAR concentration and distribution in the dendritic membrane or their conductance and Ca^{2+}-permeability are altered.\textsuperscript{150}

The second important iGluR is the NMDA (abbreviation for: N-Methyl-D-aspartate) receptor (NMDAR). In some parts of the CNS, only one type of iGluR can be found. In most areas, though, both AMPARs and NMDARs are present. In contrast to AMPARs, NMDARs lead to a much slower, but longer lasting excitatory transmission. During resting potential, the NMDAR pore is blocked by an Mg^{2+} ion. As for all ion channels in general, the Mg^{2+} blockage should not be understood as an absolute condition, since the Mg^{2+} is rather flickering in and out of the pore with altered probabilities to be either inside (i.e. blocking) or outside, depending on membrane polarisation.\textsuperscript{152}
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After binding of glutamate to the NMDAR, the channel is still blocked by Mg\(^{2+}\). To allow channel opening, an additional depolarisation, which forces the Mg\(^{2+}\) ion to exit the pore, is necessary. This essential depolarisation can be caused by previous AMPAR activation followed by Na\(^+\) ion influx. For this reason, NMDARs are commonly regarded as classical *coincidence receptors* with two factors being necessary for their activation.\(^{152}\)

![N-methyl-D-aspartic acid (NMDA) receptors](image)

*Figure 51: N-methyl-D-aspartic acid (NMDA) receptors are another type of ionotropic glutamate receptors widely distributed in the CNS. They are built up as tetramers of different subunits (NMDAR1, NMDAR2A, NMDAR2B, NMDAR2C, and NMDAR2D). Similar to AMPARs, each NMDA receptor subunit consists of four transmembrane domains (TM1-TM4). Worth mentioning, the TM2 domain does not fully traverse the membrane. During resting potential, the NMDARs are blocked by Mg\(^{2+}\) within the channel pore. As a coincidence receptor, the channel is opened by both glutamate binding and depolarisation of the membrane.*\(^{153}\)

Similar to iGluRs in general, NMDARs exist in a number of splicing variants that are responsible for different C- and N-termini mostly of the subunit GluN1. Depending on the developmental stage and the brain area, most NMDARs are heteromeric combinations of the subunit GluN1 and GluN2 (A-D). Quite recently, new subunits – GluN3A and B – have been discovered, but their role and distribution is not fully elucidated.\(^{154}\) In addition to glutamate, the subunit GluN1 accepts the amino acid glycine as co-agonist. It is still conversely discussed, whether glycine is essential or auxiliary for the receptor activity. Furthermore, GluN2B has a binding site for polyamines to allow for additional functional modulation.\(^{155,156}\) In contrast to AMPARs, NMDARs are unspecific cation channels, which not only transport Na\(^+\), but also Ca\(^{2+}\) ions. As Ca\(^{2+}\) is an important second messenger, a number of signalling pathways can be initiated in addition to the excitatory post-synaptic signal. Among others, Ca\(^{2+}\) ions are able to bind to calmodulin (CaM). Some other kinases as well as the neuronal calcium sensor *hippocalcin* are activated by Ca\(^{2+}\).\(^{157,158}\) As most of these processes and signalling pathways are of special importance during memory formation, they are further elucidated in the following subchapter.

The third iGluR type is the so-called kainite receptor. Traditionally, kainite and AMPA receptors are combined as "non-NMDA receptor family", even though the signal transmission of kainite receptors is slower than the one of AMPARs. Nevertheless, these two iGluRs share structural characteristics such as sites of extensive splicing and two RNA editing sites, which lie adjacent to TM I and TM II. Until recently, not much has been known about the distribution of kainite receptors or their role in synaptic plasticity, e.g., due to the lack of suitable antibodies.
Evidence has been found for the function of kainite receptors during learning processes, especially in the CA3 hippocampal area. While the concentration of AMPARs is usually increased during memory formation, the number of kainite receptors seems to be reduced.\textsuperscript{159,160} As kainite receptors still require extensive research regarding their detailed role in memory formation, and as some observations are not fully understood yet, they will not be further discussed here.

![Figure 52: Kainate receptors are built from multimeric assemblies of GluK1-3 and GluK4,5 subunits resulting in either low-affinity or high-affinity receptors for kainate.\textsuperscript{161} Like the other iGlurS, which have been discussed previously, they possess an extracellular N-terminal domain (NTD) that forms the ligand binding domain (LBD). Similar to AMPARs, kainite receptors undergo RNA editing and different splice variants of GluK1, 2, and 3 exist. Like this, a large number of different variants with differing functional properties and surface expression patterns exist that have not yet been fully understood.\textsuperscript{162}](image)

The Molecular Mechanisms of Synaptic Plasticity

On a molecular level, memory formation and the processes during learning are changes in neuronal transmission paths and networks. Activity-dependent changes within these networks are also known as \textit{synaptic plasticity}. The two most important forms of synaptic plasticity are referred to as \textit{long-term potentiation} (LTP) and \textit{long-term depression} (LTD) and represent an activity-dependent increase or decrease in synaptic strength, respectively. In contrast to short-term changes, which last from seconds to minutes, LTP and LTD derived changes last from minutes to a lifetime.\textsuperscript{163} Before the molecular basis of synaptic plasticity will be discussed, it should be noted that there are also two additional plasticity forms that are important for neuronal function. First, there is the so-called \textit{homeostatic plasticity}, also known as \textit{synaptic scaling}. This plasticity is a negative feedback mechanism. During LTP, synaptic transmission is strengthened. By down regulation, the neuronal firing is stabilised and kept within a physiological range. In the case of LTD, the weaker transmission is slightly up regulated. Like this, the brain prevents overshooting signals from LTP or signals that get lost within the background noise after LTD down regulation.\textsuperscript{163} Secondly, in 1996 Abraham and Bear described the phenomenon of \textit{metaplasticity} – sometimes also referred to as \textit{plasticity of synaptic plasticity}. Certain stimulation frequencies
of a neuron do not necessarily lead to synaptic plasticity, depending on the “history” of this cell. So the process of learning should not be considered as a fixed process, but rather depends on previous experiences.\textsuperscript{164}

Generally, memory formation can be regarded as changes of the neuronal network.\textsuperscript{151} Having a closer look at dendrites of excitatory synapses within the mature brain, mushroom-shaped protrusions can be observed, so-called \textit{dendritic spines}.\textsuperscript{165} The bulbous enlargements of these spines are called spine heads and are the location of glutaminergic excitatory inputs.\textsuperscript{166} Spine heads are considered biochemically and electrochemically isolated, as their neck can act as diffusion barrier with a diameter of down to 0.2 µm.\textsuperscript{165,167} In the 1970s, an electron dense area beneath the spine head membrane was observed by electron microscopy (EM) – the so-called \textit{post-synaptic density} (PSD).\textsuperscript{165} It could be shown that this mesh-like structure changes its morphology and size during phases of altered signal transmission and this allows for more efficient EPSC transmission.\textsuperscript{167,168} In addition, spine necks become wider during increased excitatory signal frequencies and consequently reduce the barrier between spine head and dendrites.\textsuperscript{167}

Later on, the content of the PSD was further elucidated. It consists of a network of different membrane receptors, ion channels, and scaffolding proteins, as well as the cytoskeleton, mostly actin.\textsuperscript{169–171} Activity-dependent morphology change is one of the key elements during synaptic plasticity and therefore the presence of actin in the PSD is of special relevance. Especially the fact that spine heads become wider and even new spines arise during LTP depicts the significance of structural rearrangement. A positive correlation between spine head volume, PSD area, and synaptic strength has been proven.\textsuperscript{165}

Interestingly, spines are hardly found in the developing brain. Instead, long, thin, and headless \textit{filopodia}, which often lack the PSD area, are observed. Due to their morphology, filopodia are more flexible in searching for appropriate binding partners, and this flexibility is of special importance in the immature brain, as the probability to find a binding partner is much lower.\textsuperscript{166} Filopodia are transient structures with a life span of minutes to hours.\textsuperscript{166,172,173} Only 10\% to 20\% of the initially formed connections get later stabilised.\textsuperscript{166} The preliminary stabilised structures are sometimes referred to as \textit{learning spines}, which are still thinner and elongated. During learning and memory formation, the spine heads become enlarged by actin cytoskeleton reorganisation. Maturing spines acquire a PSD containing all necessary proteins that are important for signal transmission. While most spines arise from filopodia in the early development, small spines without PSD can also grow in a filopodia–independent manner during later stages in the mature brain.\textsuperscript{166} Remarkably, abnormal spine structures are closely related to diseases such as Fragile X or Down syndrome that are both causes of intellectual disability.\textsuperscript{174}

![Figure 53](image.jpg)

\textbf{Figure 53:} a) Through actin polymerisation, the tube-like filopodia grow, until they have contact with an axon of another neuron. Synaptic contacts between axons and dendritic filopodia are thought to trigger the morphological rearrangement of dendritic filopodia to mushroom-shaped spines.\textsuperscript{175} b) The post-synaptic density (PSD) is a protein dense area in the post-synaptic membrane that can be visualised using electron microscopy. The PSD is proposed to contain all necessary proteins for signal transduction and morphological rearrangement.\textsuperscript{176}
LTP can be triggered, when dendrites are exposed to excitatory signals. As soon as excitatory stimulations at synapses occur, the Ca$^{2+}$ influx, mediated by NMDARs, induces LTP. In vitro, this can happen by electrical stimulation with a sequence of 100 Hz-pulses with a break of 200 ms in between. Apart from obvious morphological changes such as spine head enlargement and increase in PSD area, the actin polymerisation is significantly altered. Under basal conditions, there is an equilibrium between the globular monomeric G-actin and the filamentous polymerised F-actin. During LTP, the spine growth is caused by an equilibrium change towards F-actin. F-actin is the most important framework component within dendritic spines and the PSD, respectively, as it is also essential for some forms of vesicular trafficking within neurons.

![Figure 54: Role of actin rearrangement during synaptic plasticity. (a) Under basal conditions, there is an equilibrium between polymerised F-actin and monomeric G-actin. (b) During long-term potentiation the equilibrium is altered, F-actin becomes more stable, while depolymerisation slows down. (c) Due to the fact that polymerisation takes place at the periphery of dendritic spines, spine size increases.](image)

In addition to neuronal morphology changes such as spine density, spine size, and dendritic branching, there are also important changes regarding protein synthesis and distribution. In particular, the trafficking and surface expression of AMPARs is heavily altered. The AMPAR subunit composition critically determines the strength of excitatory synaptic transmission. It could be shown that an increased level of GluA2 within AMPARs is positively correlated to spine growth. Especially regarding the AMPAR subunits GluA1 and GluA2, it was observed that they are synthesised locally close to the synaptic sites upon increased signal transmission. Generally, AMPAR concentration is the major determinant of synaptic strength, as the ratio of NMDAR:AMPAR changes heavily during neuronal maturation. In contrast, some studies suggest an enhancement of synaptic functions by post-translational modifications during the initial phase of LTP and that local protein synthesis is important in later phases.

The NMDAR-dependent Ca$^{2+}$ influx is the key element for LTP development, because Ca$^{2+}$ is known as an important second messenger in a number of signal cascades. Above a certain intracellular Ca$^{2+}$ threshold, Ca$^{2+}$ ions are able to activate calcium-dependent proteins such as kinases. For neuronal functions, the Ca$^{2+}$/Calmodulin-dependent protein kinase II (CaMKII) and its isoforms α and β are of special interest. First, Ca$^{2+}$ binds to the Calcium-modulated protein (Calmodulin, CaM). In total, Calmodulin contains four EF-hand motifs with
each being able to bind one Ca\textsuperscript{2+} ion. Calmodulin is often used as Ca\textsuperscript{2+} sensitiser for other proteins and enzymes that are Ca\textsuperscript{2+}-dependent, but not able to bind Ca\textsuperscript{2+} themselves. CaMKII is a multimeric Serin/Threonin-specific kinase and is amongst others important for epithelial and T-cell function, but in particular in the brain for maintaining LTP. CaMKII is enriched within the PSD of excitatory synapses after LTP induction\textsuperscript{167}. It has been shown that particularly the isoform CaMKII\textalpha{} is locally synthesised very rapidly following synaptic plasticity\textsuperscript{163}. Regarding neuronal function, misregulation of CaMKII is associated with Alzheimer's Disease\textsuperscript{184,185}. Worth mentioning, only a small amount of Ca\textsuperscript{2+}-binding CaM is necessary for activation. CaMKII subunits that have already been activated by binding to CaM are able to activate neighbouring subunits within the multimeric protein complex by autophosphorylation.

Once activated, CaMKII is able to initiate a number of complex signalling pathways by phosphorylation, i.e. the MAPK phosphorylation cascade and the transcription factor CREB. Like this, CaMKII cannot only initiate local processes, but also diffusive cascades, which are for example necessary to recruit distal AMPARs\textsuperscript{151,163}. Interestingly, LTP maintenance is dependent of CaMKII presence and its persistent activity, but inversely LTP can be artificially initiated by CaMKII injection into hippocampal brain slices\textsuperscript{136,186}. After LTP induction, CaMKII can be found anchored to the PSD. Once tightly connected to the PSD, CaMKII cannot be dephosphorylated by the protein phosphatase 2A (PP2A) anymore and thus remains active\textsuperscript{184}. Within the PSD, CaMKII is hold in place by binding to the C-terminal end of NMDAR-N2B subunits that are also able to keep CaMKII active even after Ca\textsuperscript{2+} decrease\textsuperscript{167}. Recent studies propose that this NMDAR-CAMKII complex assists in AMPAR incorporation to the membrane\textsuperscript{188}. Additionally, the NMDAR-CaMKII complex is thought to bundle newly synthesised F-actin filaments and thus helps on changing spine morphology\textsuperscript{165}. In addition to the phosphorylation of transcription factors such as CREB, CaMKII has also some mechanistic roles within the PSD. By phosphorylation of the GluA1 subunit, AMPARs become more sensitive and generally show a higher conductance\textsuperscript{136}. Studies have also
shown that CaMKII is important for AMPAR trafficking and their tight anchoring to the membrane.\textsuperscript{188,189} The active amount of AMPARs in the spine head membrane can be increased during LTP via several routes. Apart from \textit{de novo} synthesis, new AMPARs, which have been "stored" in endosomes, can be released by exocytosis.\textsuperscript{190,191} Usually, under basal conditions, most AMPARs are already present at peri-synaptic sites. During LTP, AMPARs diffuse laterally to the spine membrane and are then anchored to actin within the PSD.\textsuperscript{192,193} Apart from CaMKII, there are a number of additional proteins necessary for LTP. Interestingly, aberrant AMPAR trafficking has been shown to be related to Alzheimer’s Disease, as soluble β-amyloid particles disrupt AMPAR trafficking complexes.\textsuperscript{151} As mentioned previously, the C-terminus of GluA2 has a PDZ domain for protein interaction. Most interaction partners assist in localisation and AMPAR trafficking, as it is important during synaptic plasticity. The \textit{N-ethylamide sensitive factor} (NSF), for instance, regulates vesicle transfer between membranes and like this mediates the transport from AMPAR containing endosomes to the membrane. \textit{Hippocalcin} is a neuronal Ca\textsuperscript{2+} sensor and binds to \textit{AP2}, which keeps multimeric proteins close to the membrane and assists on internalisation of transmembrane receptors via a clathrin-mediated pathway. In contrast, \textit{PICK1} also binds to membrane proteins via PDZ domain interaction and is responsible for AMPAR internalisation by binding to GluA2 during LTD. In the presence of NSF, though, this complex is disrupted. For this reason, NSF promotes AMPAR expression during LTP by several ways of action.\textsuperscript{151} Worth mentioning, the AMPAR subunit composition is altered heavily during synaptic plasticity. Following phosphorylation of AMPAR subunits by CaMKII or other kinases such as protein kinase C (PKC), Ca\textsuperscript{2+} permeable (CP) AMPARs are recruited from the peri-synaptic membrane to synaptic spines. As mentioned before, these CP-AMPARs either lack the GluA2 subunit at all or contain an unedited, glutamine containing version (GluA2(Q)). Like this, Ca\textsuperscript{2+} influx can additionally be increased during phases of heavy excitatory signal transmission. During later steps of LTP, these CP-AMPARs are replaced by Ca\textsuperscript{2+-impermeable ones to stabilise the synaptic connection. This is one of the most prominent examples of synaptic scaling.\textsuperscript{151}

\textbf{Figure 56:} During synaptic plasticity, particularly AMPA receptor composition and concentration in the region of dendritic spines are heavily altered. During LTP, the amount of AMPARs can be increased at the post-synaptic density through a number of processes such as lateral diffusion or increased exocytosis of recycled AMPARs within endosomes. Even local \textit{de novo} synthesis might be possible.\textsuperscript{151}
In contrast to biochemical processes during LTP, LTD is characterised by a sub-threshold \( \text{Ca}^{2+} \) concentration that leads to dephosphorylation and, for instance, internalisation of AMPARs. Interestingly, some kinases, such as the protein kinase C (PKC) also phosphorylates the GluA2 and GluA1 subunit at certain sites that disrupt the glutamate receptor anchoring protein (GRIP) complex and consequently lead to internalisation of the receptor. This internalisation is mediated by the mobilisation protein PICK1.\textsuperscript{163} Internalised AMPARs are either recycled during the next LTP or undergo lysosomal degradation.\textsuperscript{151,183}

Summarised, during synaptic plasticity the structural and biochemical changes rely partly on the \textit{de novo} synthesis of proteins close to the dendritic spines that have been activated before. Usually, proteins are expected to be synthesised in the soma and then transported to the location of action actively or by diffusion. For polar cells such as neurons, it has already been proven over the last decades that mRNA is transported to allow for spatio-temporal protein synthesis at locations of altered signal input.\textsuperscript{106}

By now it is widely accepted that local protein synthesis and translational control is required for memory formation. In 1965, Bodian et al. detected ribosomal particles in proximal dendrites\textsuperscript{194} and a couple of years later Steward and Levy could even detect polyribosomes in distal dendrites.\textsuperscript{195} Later on, Feig and Lipton observed incorporation of radiolabelled amino acids in the dendrites within a time frame that seemed too short to derive from transport of radioactive proteins from the soma to the dendrites.\textsuperscript{196} Finally in 1996, it was shown that synaptic transmission, which is dependent on immediate protein synthesis, could be successfully induced by neurotrophins, even though the CA1 dendrites transmitting between CA1 and CA3 within the hippocampus had been surgically separated from their cell bodies.\textsuperscript{197}

Behavioural proof of local protein synthesis is still difficult. In 2002, Miller et al. worked with mice that were lacking the 3’-untranslated region (UTR) of CaMKII including the dendritic targeting region. These mice showed rather bad results in memory and learning experiments such as the \textit{Morris water maze}. These findings are difficult to interpret, because the derivatised mRNA was present during all developmental steps and not only during learning.\textsuperscript{198}

Several methods have been applied to visualise and quantify the mRNA localisation within a cell. Apart from mechanically separated dendrites that are still able to produce newly synthesised proteins there are also other biochemical methods. Pharmacological inhibitors of protein synthesis can be used to investigate the consequences within dendrites. This approach, though, is clearly disadvantageous, since the effect might also result from the global inhibition.\textsuperscript{197,199}

Miller et al. (2002) prevented dendritic targeting of CaMKII mRNA by changing the dendritic targeting sequence within its 3’-UTR. For visualisation purposes, CaMKII was fused to GFP, so that the local synthesis of CaMKII could be visualised.\textsuperscript{198}

Local protein synthesis in polar cells has several advantages. During phases of increased transmission at certain synapses, necessary proteins become available to exactly these synaptic sites.\textsuperscript{183} Studies have already proven the presence of polyribosomes located within the dendritic shaft of spines.\textsuperscript{200} It is subject to further discussion, whether two adjacent synapses can act fully independently.\textsuperscript{183} Several studies have already shown that LTP can spread to neighbouring synapses.\textsuperscript{201–203} Nevertheless, other studies controversially discuss that particularly during the initial LTP phase (approx. one hour after induction) most changes in synaptic function rely on post-translational modifications and not on \textit{de novo}
protein synthesis. Alternatively, additional receptors, which are needed at the spine heads, can diffuse laterally from peri-synaptic areas. Especially for AMPARs, this has been proven.\textsuperscript{151}

**MicroRNA and its Role in Synaptic Plasticity**

In recent years, the importance of so-called *non-coding regions* within the genome, which are not transcribed into mRNA for protein biosynthesis, has become evident.\textsuperscript{204} The non-coding regions are divided into several groups. Transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), or small nuclear RNAs (snRNAs) are the best understood ones and referred to as *housekeeping RNAs*. Long non-coding RNAs are mostly found within the nucleus, but their role has not yet been fully elucidated except for the fact that they seem to play a role in the transcription process. Some long RNAs are also processed to shorter sequences that are able to exit the nucleus. One important representative is the so-called *microRNA* (miRNA) that has been more and more intensively investigated for the last decade and will be discussed in the following subchapter in more detail. First evidence for miRNA function raised in 1993 by Ruvkun and Ambros. They identified a gene LIN-4 as a regulator for the protein LIN-14 in *C. elegans*. The according gene products are two short RNAs that are complementary to the 3'-UTR of the LIN-14 mRNA.\textsuperscript{205,206} In 2000, another small non-coding RNA, called let-7, was found as an important regulator in *C. elegans* development.\textsuperscript{207} This group of regulatory RNAs was later called *microRNA* (miRNA) and is known for almost all organisms by now. Within the genome, miRNAs are found mostly in separated miRNA transcription areas, but also within introns. After transcription by the polymerase II, the so-called *primary miRNA* (pri-miRNA) contains the structural characteristics for transcription products such as capping and polyadenylation. The pri-miRNA has a stem-loop structure and a size of up to 1000 nucleotides (nt). Within the nucleus, the pri-miRNA is then digested by the type-III RNase Drosha. Together with the double-stranded RNA (dsRNA) binding protein DGCR8, Drosha produces the hairpin structured *precursor miRNA* (pre-miRNA) with a size of 60-70 nts. The pre-miRNA is a typical type-III RNase product with a 2-nt overhang at its 5'-end and can exit the nucleus into the cytosol, where the pre-miRNA is then further processed by another type-III RNase called Dicer to the mature double-stranded miRNA. Similar to Drosha, Dicer is assisted by a dsRNA binding protein TRBP (abbrev.: transactivating response RNA-binding protein).\textsuperscript{208} This cofactor is also responsible for the recruitment of AGO2, a central component of the RNA-induced silencing complex (RISC). Within the initial microRISC loading complex, AGO unwinds the double-stranded miRNA. The strand with the less stable base pairing at the 5'-end is loaded into the miRISC and functions as guide strand.\textsuperscript{209} The human member of the argonaute family, AGO2, is the centre of RISC and is related with translational repression among other auxiliary proteins.\textsuperscript{210} It is also the only AGO form that has endonucleolytic activity, if the target mRNA is fully complementary.\textsuperscript{211} These findings are similar to the ones made for siRNA.\textsuperscript{212} Depending on the degree of complementarity, target mRNAs of the mature miRNA can either be degraded or their effective initiation of translation is prevented by decapping or deadenylation. The experimental finding that AGO2 does not always cleave the target mRNA is still heavily discussed. Some researchers believe that the silencing is based on complete deadenylation and finally decay. Others suggest that reversible translation repression might also play an important role.\textsuperscript{212} In mammals, translation repression is the dominant effect due to
incomplete base pairing between miRNA and target mRNA. Despite the acceptance of incomplementarity in parts, the nucleotides 2-8 at the 5'-end, which are called seed region, require perfect base pairing, mostly within the 3'-UTR of the target mRNA.\textsuperscript{213,214} Worth mentioning, some miRNAs can even bind within the coding region of mRNAs\textsuperscript{215} or at the 5'-UTR.\textsuperscript{216}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{miRNA_production}
\caption{The production of mature miRNA basically consists of two intermediate products. In a first step, the primary miRNA transcript (pri-miRNA) is produced by RNA polymerase II or III. The pri-miRNA is then cleaved by the microprocessor complex Drosha-DGCR8 in the nucleus. The resulting precursor stem-loop structure, the pre-miRNA, is exported from the nucleus by Exportin-5. In the cytoplasm, the RNase Dicer further processes the pre-miRNA hairpin to its mature length. This happens in complex with the double-stranded RNA-binding protein TRBP. The mature miRNA is loaded into the RNA-induced silencing complex (RISC). Argonaute (Ago2) proteins are essential parts of this complex. RISC is able to silence target mRNAs by mRNA cleavage, translational repression, or deadenylation. The passenger strand is degraded.\textsuperscript{217}}
\end{figure}

Due to the sufficient partial complementarity except for the seed region, one miRNA can have several hundred target mRNAs, even though not all interactions are necessarily physiologically relevant.\textsuperscript{212} As it has been discussed in the previous subchapters, polar cells such as neurons require local, activity-dependent protein synthesis. Especially in hippocampal neurons, de novo protein biosynthesis is required for long-term memory formation.\textsuperscript{214} Some proteins might even be harmful when synthesised at wrong locations. One example is the myelin basic protein (MBP), a component of the axon-wrapping Myelin sheets. This protein would interact tightly and unspecifically with the soma membrane when translated at the wrong position within a cell.\textsuperscript{218} Therefore, regulation of neuronal activity and this tight spatio-temporal control of protein synthesis are of special importance for proper function. Uncontrolled excessive activity is related to neuronal cell death. During stroke, for instance, unphysiologically increased cell depolarisation and a resulting unpysiological Ca\textsuperscript{2+} concentration, which cannot be compensated, is observed.\textsuperscript{216}
It is still unclear how mRNAs are prevented from being translated at wrong locations within a cell. Intracellularly, mRNAs are packed in P-body like granules. Within the granule, the microRNA might keep the mRNA within a dormant state, until translation is needed to provide a spatio-temporal control of translation. These granules are then transported within the neuron. The detailed composition of these granules remains still unclear. Some studies suggest a number of mRNAs per granule, while others suggest the presence of a single one.\textsuperscript{106} Even though there are no clear evidence for miRNAs working in a spatio-temporal manner or for their presence within the mRNA granules for temporal translation inhibition, there is an increasing number of studies that support this hypothesis.\textsuperscript{212} Several miRNA-knock-out mice show impaired long-term memory formation, even though it is technically difficult to investigate the brain-specific role of miRNAs, as there might not be a clear phenotype and it is generally challenging to suppress miRNA expression locally.\textsuperscript{217} Recently, it has been shown that mRNA is sequestered by RNA-binding proteins (RBPs) directly after transcription.\textsuperscript{216} Regarding the directed transport of dormant mRNA, there are different hypotheses present. For some dendritic proteins such as MAP, CaMKII, AMPAR, or NMDAR subunits, certain sequences have been identified within the 3'-UTR of the respective mRNA that function as zipcodes. These cis-acting elements might interact with RBPs for specific transport. β-actin mRNA, for instance, show axon-directed cis-acting elements.\textsuperscript{219–221} These zipcode-like sequences might be used for direct transport along the cytoskeleton. Alternatively, the mRNA granule diffuses and is locally anchored. During transport within the granule, mRNA must be protected from degradation.\textsuperscript{222–224} Dendritically targeted mRNA and granules, respectively, are better studied, even though also axon-directed transport is known.\textsuperscript{106,225} The presence of mRNA in axons has long been unknown, as translational machineries have hardly been found.\textsuperscript{216} Nevertheless, not all miRNAs might be found within granules directly after mRNA transcription. Some miRNAs are especially enriched at axons, dendrites, or even more specifically exclusively close to synapses within dendritic spines.\textsuperscript{226–229} Additionally, there is growing evidence, that miRNA maturation might also be locally controlled.\textsuperscript{227,230} One well-studied example of a prototypic dendritically located miRNA is miRNA-134. Bickler et al. could show that even the pre-miRNA-134 is localised specifically in dendrites by using a fluorescently labelled pre-miRNA-134. They could even identify the targeting mechanism. It is known that RBPs sequence-specifically interact with the terminal loop of pri- and pre-miRNAs and it was hypothesised that the loop sequence determines localisation.\textsuperscript{231,232} For miRNA-134 and its respective pre-miRNA-134, Bickler et al. could show that the DEAH box helicase DHX36 binds sequence-specifically to the loop and guides the pre-miRNA to the dendrites in contrast to sequences with the loop sequence of the somatically located pre-miRNA-150.\textsuperscript{233} It can be hypothesised that, by binding to the loop sequence, DHX36 protects the pre-miRNA-134 from being processed by Dicer;\textsuperscript{216} Regarding miRNA-134 function, Bicker et al. could show in an additional study that its target is the mRNA of LIMK1. The resulting protein LIMK1 is a kinase that is important for brain development and positively related to dendritic spine growth upon BDNF exposure.\textsuperscript{163} These findings are consistent with the fact that mature miRNA-134 is found in close proximity to synapses of postsynaptic cells.\textsuperscript{163,233} By inhibiting LIMK1 translation, actin polymerisation is prevented, so that spine size and therefore synaptic strength is decreased.\textsuperscript{217,225,234} Consequently, miRNA-134 overexpression leads to LTP impairment in mice.\textsuperscript{212} Interestingly, the transcription factor CREB was also found to be down regulated.\textsuperscript{225} CREB has several miRNA binding sites within its 3'-UTR, e.g. for miRNA-134 and miRNA-124, but in turn CREB is also responsible
for the respective pri-miRNA transcription by binding to the according promoter region as a 
negative feedback mechanism. This might also be an important mechanism similar for 
synaptic scaling to prevent uncontrolled activity, as it is observed in neuropsychiatric 
disorders.

Even though the loop sequence seems to be important for localisation for some miRNA 
precursors, it is still unknown how pre-miRNAs are reliably prevented from being 
processed in inappropriate locations. Some studies suggest several activity-dependent 
control elements. MiRNA turnover rates or the degradation and production, respectively, of 
RISC components can be adapted to neuronal signal transmission. RISC remodelling, in 
particular, seems to be both growth factor (i.e. BDNF) and Ca\textsuperscript{2+}-dependent. Membrane 
depolarisation causes ubiquitination of some RISC-associated proteins, so that kinases for 
signal transmission such as CaMKII\textalpha and LIMK1 are efficiently translated. Additionally, 
Dicer processing of the respective pre-miRNA could be controlled in an activity-dependent 
manner. Especially the latter possibility requires further research. It is not yet 
understood why neuronal activation causes Dicer inhibition or activation depending on the 
regarded miRNA precursor. Most hypotheses that are currently under discussion do not 
sufficiently explain why and how some miRNAs are synthesised more efficiently during LTP, 
while the amount of others is massively reduced. MiRNA-134, for instance, causes spine 
shrinkage due to impaired actin polymerisation, while miRNA-138 leads to spine growth 
during LTP. Other examples are miRNA-132 that impairs novel object recognition when 
overexpressed. In contrast, the presence of miRNA-195 increases spatial memory and 
orientation in mouse models. Obviously, there are further regulators of miRNA function. 
Presumably, RBPs might compete with miRNAs for 3'-UTR binding sites. Recent studies have explored another RNA species – circular RNA (circRNA) that functions 
as miRNA sponge and sequesters miRNAs. One example is the circRNA ciR7 that has 73 
binding sites for miRNA-7.
Motivation and Research Aims

Nucleic acid chemistry has become more and more important within the last decades. Especially the progress in solid-phase synthesis made highly modified oligonucleotides available for therapeutic applications and basic research interests. Furthermore, inventions such as the polymerase chain reaction (PCR) by Mullis in 1983\textsuperscript{236} or the optimisation of sequencing techniques by Sanger et al. in 1977\textsuperscript{237} helped to understand and decipher the genetic code with all its buildings blocks. The detailed elucidation of the RNA interference (RNAi) mechanism by Fire and Mello\textsuperscript{238} allowed for further understanding of post-transcriptional gene regulation. Besides, RNAi has been shown to be a very promising therapeutic tool regarding specifically targeted gene therapy in diseases such as cancer or genetic disorders. The antisense oligonucleotide (ASO) therapeutic approach has made promising progress within the last years and it might become possible to treat disorders such as Amyotrophic Lateral Sclerosis (ALS).\textsuperscript{239}

Regarding basic scientific research, the elucidation of local processes within living cells has become of large interest. Especially transport mechanisms and the specific targeting of RNA is not understood yet. Additionally, it is still unknown why some enzymatic processes are restricted to certain localisations within a cell.

In the present PhD Thesis, two approaches will be introduced that allow for investigation of locally restricted processes within a cellular environment \textit{in vivo}.

In the first project part, the focus lies on Molecular Beacons. Molecular Beacons are fluorescently modified oligonucleotides that are present in a stem-loop structure in the absence of their complementary target sequence. Upon binding to their target sequence within a cell, this closed stem-loop structure is opened up and the resulting fluorescence can be used as read-out.

![Figure 58: Principle of common Molecular Beacons. Due to their structural design, they stay non-fluorescent, until the complementary target sequence forces the structure to open up.](image)

In most cases, commonly used Molecular Beacons result in a rather high background fluorescence, as the respective cellular target sequence is usually present in high concentrations and evenly distributed within a cell, not only at the location of interest. Additionally, nuclease-dependent digestion of Molecular Beacons leads to false positive results and further increases out-of-focus fluorescence. To overcome these issues, it would be desirable to use extremely low intracellular probe concentrations that are difficult to achieve experimentally. Besides, low probe concentrations decrease the probability of the probe being located at the localisation of interest in sufficient amounts. Instead, it would be
ideal to have a probe design that allows for spatio-temporally controlled activation of the probe in areas of interest.

In this case, we used light-inducible Molecular Beacons that can be made detection-competent by the use of spatially defined laser pulses. Former light-inducible designs showed a number of drawbacks such as incomplete inactivation, a fact that causes low signal-to-noise ratios. Other early design from the group of HECKEL et al. needed up to seven photolabile protecting groups to achieve full inactivation of the Molecular Beacon. Like this, it is almost impossible to ensure full deprotection or exclude irreversible side reaction of the photolabile protecting groups.

In our improved design, we indirectly inactivate the fluorophore instead of the Molecular Beacon itself. The fluorophore is coupled to a quencher via a photolabile linker. The choice of the linker length and the distance between the dyes, respectively, was a very critical step. It was not clear either, whether all fluorophore-quencher pairs are suitable for this design due to their chemical characteristics and individual interactions. Furthermore, it must be noted that most dyes are not photochemically stable enough for tracking experiments, as the used laser pulses are rather high and most dyes tend to bleach quickly. For this reason, initial approaches were made to couple photostable quantum dots, which had previously been inactivated by photoresponsive aromatic systems, to Molecular Beacons. This approach using photoinducible quantum dots was very challenging, as the labelling strategy of quantum dots differs massively from common organic dyes and had to be optimised carefully.

In a second project we desired to visualise local enzyme activity within neurons. In recent years, it has been shown that microRNA plays an important role in post-transcriptional gene regulation in mammals. Especially in polar cells such as neurons, processes such as messenger RNA translation and its microRNA-dependent regulation are hypothesised to occur in a spatially and temporally defined manner. In particular, memory and learning processes and their molecular basis, referred to as synaptic plasticity, are thought to underlie these locally restricted processes.

Therefore, the aim was to develop probes that mimic the native microRNA precursor pre-microRNA-181a1, which is of special interest in brain function and synaptic plasticity. Upon processing to the mature microRNA by the endonuclease Dicer, the designed probe starts to fluoresce and the local signal could be detected in real-time.

![Figure 59: After primary Drosha cleavage, the resulting stem-loop-structured pre-miRNA is processed by the endonuclease Dicer to yield the mature miRNA duplex. The aim of this study was the preparation of a fluorescently modified pre-miRNA-181a1 and to visualise its locally restricted maturation. The probe was supposed to fluoresce after Dicer-mediated processing (Q: quencher, F: fluorophore).](image)

Both projects included solid-phase synthesis, in particular the synthesis of very long and highly modified RNA sequences. These techniques had to be established in the working
group. Especially the positions that are accepted in vivo and the respective labelling strategy needed to be carefully chosen. The introduction of several apolar modifications into an oligonucleotide is known to make purification and handling more and more challenging. The choice of suitable fluorophore-quencher pairs and their respective labelling strategy is a critical step for further studies such as single-molecule imaging.

The synthesised probes for both projects needed to be purified using different chromatography techniques depending on the type of oligonucleotide and the chosen modifications. Possible techniques included reversed-phase, anion exchange, or size exclusion chromatography. The probes also needed to be characterised and functionally tested in vitro for desirable signal-to-noise ratios, chemical stability, and proper function for further in vivo experiments.

After final in vitro testing, the respective probes were applied in cell culture for either single-molecule imaging in Chironomus tentans cells for the first Molecular Beacon project, while we chose to work with hippocampal rat neurons for visualisation of microRNA maturation in the second project.

The first part of the projects including chemical synthesis, purification, analysis, and initial tests (e.g. in vitro fluorescence tests) were performed during this PhD Thesis in the group of Prof. Dr. Alexander Heckel.

Further in vivo experiments including a number of microscopy techniques were either conducted by the group of Prof. Ulrich Kubitscheck from the University of Bonn for the MB project or by the group of Prof. Erin Schuman from the MPI for Brain Research, Frankfurt, for the miRNA project.
General Part

Feedback Tracking

For a number of years, it has been known that mRNA translation does not occur globally within the whole cell. Particularly polar cells are dependent on an asymmetric protein distribution, which is based on protein biosynthesis at well-defined sites within the cell. In contrast to the majority of membrane-protein-coding mRNAs, it is not yet fully understood what prevents certain mRNAs from being translated anywhere within the cytosol. It neither has been elucidated what determines the specific transport of these mRNAs. The advantage of on-demand protein biosynthesis is the easier transport of dormant mRNAs within mRNA-proteins (mRNPs) to certain locations within a cell where it can be translated several times. As already mentioned in the introduction part, the memory formation in certain brain areas such as the hippocampus relies on those locally restricted processes. But not only transport processes of mRNA are still under research. Also the detailed processes during nuclear export of the mature mRNA such as folding state, recognition pattern of mature mRNA, and intracellular mRNA processing need further studies. Processes like these can only be understood, if single molecules are tracked in real time in living cells under native conditions. Preliminary results by KUBITSCHEK et al. elucidated the export processes of a single mRNA through the nuclear pore. The group chose to use the model organism Chironomus tentans. By using fluorescently labelled ribonuclear particles, the trajectories of nascent mRNPs could be observed using light-sheet fluorescent microscopy (LSFM). Chironomus tentans is a well-studied model organism of choice for single-molecule RNA tracking experiments and will also be used in this thesis – in particular its salivary gland cells. The reason for this popularity is the presence of so-called polytene chromosomes – also known as Balbiani Rings (BR). These enlarged chromosomes are formed, when a cell replicates its genome without cell division, so that up to 8,000-16,000 copies of a certain gene can be produced within one single cell. As a result, the volume of the cell is massively increased. Additionally, the organism gains a metabolic advantage. The presence of several gene copies implies higher expression levels of the according mRNA and protein, respectively. Both facts make these cells an optimal candidate for mRNA tracking experiments. As shown in Figure 60, BRs form very characteristic patterns and are easily observed under the microscope due to their banded and puff-like structure.
Figure 60: Polytene chromosomes (also named Balbiani rings (BR)) have characteristic light and dark banding patterns that have been used to investigate chromosomal rearrangements and changes in transcriptional activity. Dark banding often corresponds to inactive sites, whereas light banding is usually found at areas with higher transcriptional activity. These areas also show a puffed structure with loose chromatin packing.

In *C. tentans* this phenomenon can be found prior to pupation. During this developmental step, a high copy number of a glue-like protein is needed. By observing these mRNAs and their fluorescently labelled particles, KUBITSCHEK et al. successfully demonstrated that only a minority of mRNPs are directly exported. The group rather observed a long dwell time at the nuclear pore complex. This finding might indicate the presence of a last quality control checkpoint within the nuclear basket to prevent immature pre-mRNA from being exported and translated.

Figure 61 above depicts a single hrp36-labelled mRNP during its export from the nucleus into the cytoplasm. AlexaFluor647-labelled hrp36 molecules were injected into the cytoplasm, transported into the nucleus, and finally integrated into mRNPs. Like that, the group could follow single hrp36-AlexaFluor647-labelled mRNPs in the gland cell nuclei in a depth of about 120 µm within the sample. To make sure that each mRNP contained maximally one fluorescent hrp36, the concentration of injected hrp36 was extremely low.
Precise live images like this can only be achieved by using LSFM. In these applications, the common fluorescence microscopy is not suitable, as the whole cell lies within the exciting laser beam. As a consequence, all fluorophores within the cell are excited, even though only a small fraction lies within the focus of the microscope. As a result, the background fluorescence is usually higher causing a higher detection limit and shorter tracking times due to untimely fluorophore bleaching. By using LSFM, exclusively a very thin sheet of sample is excited exactly at the focal plane of the microscope. This technique was first described by Stelzer et al. in 2002. With this microscopy technique, the background due to out-of-focus fluorescence can be drastically decreased. Depending on the wavelength, the light-sheet can be as thin as 2-3 µm. Due to the movement of the sample, a high-speed camera keeps the objects of interest within the focus and thus follows the pathway of the molecules.

![Image](image.png)

**Figure 62:** In light sheet fluorescence microscopes, the illumination is done perpendicularly to the direction of observation. The laser beam is focused in one direction by a cylindrical lens. Like this, a thin sheet of light is created in the focal region that can be used to excite fluorescence only in a thin slice (in the range of µm depending on the wavelength) of the sample. The fluorescence light emitted from the light sheet is then collected perpendicularly with a standard microscope objective and projected onto a detector such as a CCD camera.

In a recent study, the group of Kubitscheck even succeeded in real-time three-dimensionally resolved particle tracking of ribosomal and BR-derived mRNA. They could yield up to 1,000 localisations of an individual molecule. When the molecule leaves the focal plane, computational calculations make it possible to adjust the light-sheet and microscopy focus accordingly in an image-based feed-back loop. To overcome photoinstability of organic dyes, an antisense mRNA probe was used that had been labelled with three Atto647 dyes. This dye is one of the most stable ones that are commercially available to date. During the collaboration with the group of Prof. Kubitscheck, the following probes were designed and labelled with Atto647 in a post-synthetic manner using the commercially available Atto647-NHS ester.
Table 2: Sequences of oligonucleotides used in this study. The positions of amino group modifications are shown in bold. For internal amino modifications, the commercially available dT(NH2) nucleotide shown below is used.

<table>
<thead>
<tr>
<th>28S rRNA oligo:</th>
<th>5'-H₂N(CH₂)₆-linker-CAU UCG AAU AUU UGC dT(NH₂)AC UAC CAC CAA GAU CUG-NH₂(CH₂)₆-linker-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR2.1 3x oligo:</td>
<td>5’-H₂N(CH₂)₆-linker-CUU GGC dT(NH₂)UG CdT(NH₂)G UGU dT(NH₂)UG CUU GgdT(NH₂) UUG C-NH₂(CH₂)₆-linker-3'</td>
</tr>
<tr>
<td>BR2.1 1x oligo:</td>
<td>5’-H₂N(CH₂)₆-linker- ACU UGG CUU GCU GUG UUU GCU UGG UUU GCU-3'</td>
</tr>
</tbody>
</table>

Several positions within the antisense oligonucleotide of the BR2.1 mRNA and the 28S rRNA were modified with amino linkers for the terminal positions or with amino modified dT(NH₂) nucleotides for internal positions. This approach allows for post-synthetic labelling with Atto647-NHS esters, as it has been theoretically explained in the introduction. Initially, it was desired to introduce even more than three fluorophores within the BR2.1 complementary sequence, so that in total six amino modifications were introduced into the sequence. After labelling, though, the purification of the fully labelled product seemed unpromising, as too many regioisomers had been formed, as a result of incomplete labelling. Besides, even though the molecular structure of Atto647 has not yet been disclosed by the company, it is known that this dye is a mixture of two diastereomers, leading to further purification issues (cf. Figure 64)

Figure 63: Molecular structure of amino modified dT nucleotide dT(NH₂). The amino group can be labelled using dye active esters such as NHS esters. The modification is not supposed to interfere with the binding of the labelled oligonucleotide to its complementary target.

Figure 64: RP-HPLC chromatogram after Atto647-labelling of a BR2.1 oligo, which is modified with six amino groups. Only a 3x labelled oligonucleotide could be isolated.
Feedback Tracking

The HPLC chromatogram shows the difficulties in isolating the pure BR2.1 complementary sequence with up to six fluorescent labels. The presence of different regioisomers (due to incomplete labelling as well as the diastereomeric character of Atto647) makes it almost impossible to distinguish between different peaks.

MS analysis revealed that the highest yield could be achieved for BR2.1 oligos with three fluorescent labels (BR2.1 3x oligo), independently from their position. The additional amino groups are not expected to disturb binding, as they are positioned to lie inside the major groove and not to interfere with Watson-Crick base pairing. We decided to use these BR2.1 3x oligo despite the presence of additional unlabelled amino groups.

Applying three labels to a 33nt-long oligonucleotide was found not to be problematic in general, as the 28S rRNA complementary strand (28S rRNA oligo) could be purified in sufficient amounts. It could be discussed whether the distance of only 3-4 nucleotides might not be sufficient for labelling reactions with bulky xanthene-type fluorophores to be successful.

Key to feedback tracking is a photon-efficient instrument and a sensitive detection system. Therefore, the group of Kubitscheck chose light sheet illumination in combination with optimised labelling strategies that allow for specific single molecule signals with low unspecific background. The feedback loop for single molecule tracking consists of a library coupled to the instrument software that compares positional information from the image data with the library. Additionally, a piezo stage is integrated, which is used to reposition the entire sample holder based on the positional data. Like this, a particle of interest can continuously be kept within the light sheet and within the focal plane of the microscope setup.

![Figure 65: Schematic view of the 3D feedback tracking setup. Fluorescence was excited by light sheet illumination. The 3D particle position was encoded by astigmatic imaging. The positional information was used to drive a piezo stage and to keep a particle of interest close to the focal plane. Feedback tracking is based on the distortion of a particle image that can be matched to templates. This template matching can be used for axial localisation.](#)

If not limited to the cell membrane, typical intracellular trajectories in single-molecule tracking experiments contain only 10–20 localisations. This fact is due to the small axial detection range (cf. Figure 66). In contrast, feedback tracking can yield hundreds of localisations in a single trajectory.
The movement of a particle that interacts with a larger particle or cellular compartment is characterised by the absence of positional changes during the interaction time. A technique like the feedback tracking would allow for a deeper understanding of the kinetics underlying the different motion states and distribution within a cell.

Figure 66: In common tracking experiments the axial detection range (grey) restricts the duration of trajectories to the time period of the particle being within the focal plane (blue line). Feedback tracking can extend the observation time per particle up to the limit of photobleaching, as it follows the molecule or particle even during axial movement (black line).

Like this, tremendous prolongations for the observation of BR2.1 or rRNA particles could be achieved. The fluorescently labelled oligonucleotides directed against BR2.1 mRNA were microinjected into C. tentans salivary gland cell nuclei. For background staining of the nuclear envelope or the DNA within the nucleus in general, either the fluorescently labelled nuclear transport factor NTF2-Alexa546 or DNA-staining SYTOX Green was co-injected. Trajectories covering an axial range of several µm were achieved from BR2.1 mRNA particles labelled with oligonucleotides carrying a single Atto647 dye molecule (BR2.1 1x oligo). However, photobleaching limited the theoretically possible tracking time. To achieve a further prolongation of trajectories, the oligonucleotides with three fluorescent modifications were applied (BR2.1 3x oligo). As a result, a number of extremely long single particle trajectories over a time course up to 16 s were obtained. The longest observation of an individual particle consisted of 769 localisations with a total duration of 15.8 s. By dwell time analysis, KUBITSCHECK et al. observed that initially slowly diffusing particles accelerated significantly toward the nuclear envelope.

Figure 67: Tracking of BR2.1 mRNPs in the nucleoplasm. Ten trajectories with more than 200 localisations were found in a single nucleus. The grey line indicates a polytene chromosome (PC), the white line the nuclear envelope (NE) separating the nucleoplasm from the cytoplasm (CYT).
One of the trajectories (#5) overlapped with a polytene chromosome. During the time of overlap, a reduced mobility could be identified in the respective trajectory. This finding is not unexpected, because it is known that the dense packing of polytene chromosomes strongly hinders diffusion of larger particles\textsuperscript{250}. Other dwell times might result from aborted nuclear export events, during which the mRNA particle enters a nuclear pore complex but is rejected from leaving the nucleus and is released back into the nucleoplasm. Previous experiments with a static focal plane had already suggested a discontinuous motion of BR2.1 particles, but had not been proven directly\textsuperscript{250,251}.

In addition to the tracking of BR2.1 mRNA particles, it was possible to observe and analyse the dynamics of rRNA particles (rRNPs) for the first time. Oligonucleotides targeted against the 28S subunit of the rRNA were microinjected into nuclei of \textit{C. tentans} salivary gland cells. Interestingly, these particles moved much faster than BR2.1 mRNA particles most likely due to their smaller size. However more distinct immobilisation events could be observed. While mobile particles were found throughout the entire nucleus, rRNPs were mostly immobile within the nucleolus during their synthesis or during repeated interactions with the nucleolus. For the first time, it was possible to detect repeated interactions between the rRNP and the nucleolus, even though the interactions sites were axially separated. A common experimental setup with a static detection plane would have not been able to record both events in a single trajectory.

![Figure 68: Trajectory of particle #5 in the figure above. The orange background marks time periods of reduced mobility. One of these dwell times (c) can be explained with trajectory #5 overlapping with the polytene chromosomes.](image)

![Figure 69: 3D tracking of a ribosomal RNA particle. a) Full 3D rRNA trajectory b) Overlay of the trajectory with the NTF2-AlexaFluor546 signal to highlight the nuclear envelope. NTF2 was enriched in the nucleoplasm (green), but cannot be found within the nucleolus (NUC) where rRNA is synthesised. c) Time course of particle movement. The dwell times \(k_1\) and \(k_2\) correlate to times of rRNP interaction with the nucleolus. During this time, the particle seems fully immobile.](image)
With the feedback tracking method presented here, it is possible to track fluorescently labelled molecules over a time period of several seconds and obtain full spatial information for the first time. Like this, it is possible to investigate mobility patterns underlying biological processes of individual molecules. Other 3D tracking techniques usually have lower photon efficiencies, so that photobleaching limits the tracking period.\textsuperscript{252}

The possibility to follow particles even during axial movement and measure their time of immobility or their change in mobility in times of interactions with intracellular compartments might become useful for the future examination of RNA dynamics within living cells.

Oligonucleotides carrying three organic dye molecules even allowed for further increased SNR and minimal illumination intensities.

In general, further extension of tracking times for fluorescently labelled biomolecules could become useful for the understanding of particle interaction, aggregation or self-organisation phenomena.
Light-inducible Molecular Beacons – a New Approach

The techniques used by Kubitscheck and his group rely on fluorescence molecules that can bind to the RNA of interest (target RNA). As done by this group, it is possible to label proteins of the mRNP complex such as hrp36 fluorescently. Alternatively, fluorescently modified oligonucleotides, which are complementary to the RNA of interest, serve as so-called FISH probes (fluorescence in situ hybridisation, cf. introduction). It has already been discussed that approaches like these two have the disadvantage of being permanently fluorescent. Therefore, it is not possible to distinguish between the signals of the bound and the unbound probe. The read-out of the signal does not necessarily represent the distribution of the target RNA. This fact also leads to low SNRs and makes it often necessary to fix and wash the cells prior to imaging.

Neither the MS2-GFP approach is able to meet all requirements that are necessary for real-time tracking of RNA in living cells. Even if transfection and the usage of modified RNA is not primarily an issue, it should be noted that for the MS2-GFP approach, the target RNA becomes much longer. This fact and the additional binding of fluorescent GFP proteins could possibly change the movement and localisation of the target RNA.

Regarding the massive drawbacks of these techniques, the rising popularity of Molecular Beacons (MB) is easily comprehensible. As explained previously, the design of MBs with a fluorophore and a quencher at either of the two ends, keeps the MB in a non-fluorescent state, until the target sequence has bound.

Due to this mode of action, the SNR is superior to other techniques mentioned above. Depending on the design, MBs are selective up to single-mismatch discrimination. Previous disadvantages of MBs such as false positive results due to endonucleatic digestion could be overcome by the usage of DNA derivatives such as 2′-O-methyl-RNA-based MBs. As an additional advantage, modifications such as 2′-O-methyl-RNA, LNA, or PNA show a much higher binding affinity to the target RNA and can be easily incorporated depending on the desired specificity and application. Nevertheless, most fluorescent probes have in common that they result in a rather high fluorescent signal within the whole cell. This is especially true, if the amount of target RNA is rather high and evenly distributed within the cell, even though exclusively bound MBs fluoresce. Extreme low concentrations might well solve this problem, but cannot be applied reliably.
Some groups tried to solve this problem by applying photoinducible fluorochromes into antisense oligonucleotides (ASOs) and activated the dye solely at certain sites within a cell where mRNA transport was of interest.\textsuperscript{253}

Figure 71: 1) Chemical structure of light-inducible Fluorescein (green) with the light-responsive moiety (o-nitrobenzyl group) in black. The biological macromolecule is attached to the caged Fluorescein at position R1. (2) Mitotic spindle labelled with caged-Fluorescein tubulin (green in blue box) can be uncaged and the resulting fluorescent tubulin (green) followed, as it moves polewards. The figure part 3) depicts the microscopic images. The upper row demonstrates the labelling with common non-caged Fluorescein, the row below shows a massively increased SNR due to the usage and local uncaging of caged Fluorescein. The figures depict the time prior to uncaging and at two time points afterwards.\textsuperscript{253}

Unfortunately, this technique cannot easily be applied to other fluorophores. Even for related rhodamine-based dyes, which also belong to xanthene dyes like Fluorescein, it is much more complex to achieve photoinducibility. Most commercially available xanthene and cyanine-based dyes do not offer any functional groups at all for modifications in a comparable manner.

Figure 72: a) In contrast to caged Fluorescein, rhodamine dyes need to be caged with a carbamate linker in between, which is more complex to synthesise. Most modern dyes such as b) Atto565 or c) Cyanine 3 dyes cannot easily be caged due to the lack of accessible functional groups.

In 2009, the group of \textsc{Weihong Tan} tried to solve these problems of MBs by introducing the first light-inducible MB design. Their MB was characterised by a photocleavable linker that connected fluorophore and quencher at both ends of the MB covalently.\textsuperscript{240}
The linker was applied post-synthetically via Cu(I)-catalysed click chemistry. Like this, the Molecular Beacon was forced in its closed form even in the presence of its target. Fluorophore and quencher could not be separated far enough from each other to overcome the FRET distance. Even though the concept should work in principle, even before irradiation the fluorescent signal of the MB was rather high suggesting that the photolabile deactivation (caging) was not sufficient. Furthermore, full fluorescence could not be regained with 200 mW/cm$^2$ and even though the half-life of the photoreaction was found to be 0.6 s.$^{240}$

One year later in 2012, the group of Heckel et al. published an alternative design that also prevents the caged MB from binding to its complementary target sequence. In this approach, photolabile protecting groups (PPGs, in literature also referred to as cages) were covalently attached to a number of nucleobases within the loop region. Like this, Watson-Crick base pairing can efficiently be disturbed. In the following, this design will be referred to as loop-caged MB.$^{254}$
Several nucleobases within the loop region are modified with light-responsive moieties such as NPP that prevent Watson-Crick base pairing. As a consequence, the MB is not able to bind to its target until activation by light.

In her PhD Thesis, VERA MIKAT could show that the efficiency of inactivation is dependent on the number of PPGs present in the loop region. In general, five to six PPGs seemed to be sufficient for MBs with a 20nt-long loop region. Like this, MIKAT et al. could achieve off-on-ratios of up to 1:20.

Even though this design showed good SNR results, the approach has some drawbacks making it unsuitable for general applications. First, the PPGs need to be attached prior to SPS for every nucleoside phosphoramidite needed. The synthesis of caged DNA building blocks consists of approx. six generally previously published steps. Later in this chapter, the synthesis is shown for NPP-modified dG (dGNPP), as this building block has also been synthesised and used for the studies presented in this thesis. Depending on the loop sequence of the MB, it might become necessary to synthesise several caged phosphoramidites making the design time-consuming.

Another drawback of loop-caged MBs results from the fact that several PPGs are needed to ensure full deactivation. During light-dependent activation not all PPGs might be removed, also resulting in decreased specificity due to mismatch positions. Besides, as already mentioned in the introduction chapter, some PPGs such as NPP show pH-dependent, irreversible side reactions that impede full deprotection and consequently impair the specificity of the MB.

Noteworthy, only after uncaging the binding process of the MB to its target sequence can occur. Depending on the chosen building blocks and their respective affinity to RNA, the binding kinetics may vary and full fluorescent signal may only be reached after a time frame of seconds to minutes.

In this study, a well-known and optimised MB was used that is specific for the BR2.1 mRNA in salivary gland cells of C. tentans.
This MB was designed as both DNA- and 2'-O-methyl-RNA-based probe. Binding kinetics were determined by in vitro fluorescence measurements. For a 1:1 concentration ratio of MB to target RNA, the results are shown in Figure 78.

Clearly, the binding process does not happen simultaneously with the uncaging process and this fact might make it difficult to track mRNA molecules in real-time without time lapse after the uncaging process using previously published designs.

In this project, the previous drawbacks of early light-inducible MB designs should be solved. One possible solution in the reversibly photolabile inactivation of a fluorophore, similar to the caged Fluorescein applied on ASOs in FISH experiments.\(^{253}\) Even though caged Fluorescein is easily available to date, it is by far not the optimal fluorophore for tracking experiments that need a high laser power over a long time frame. Fluorescein bleaches quickly and its fluorescence is strongly pH-dependent. Additionally, a number of cells show auto-fluorescence at the excitation wavelength of Fluorescein (\(\lambda_{ex} = 485\) nm / \(\lambda_{em} = 514\) nm). To apply the idea of caged fluorophores to state-of-the-art commercially available dyes such as AlexaFluor and AttoTec dyes, published designs by Tang AND Dmochowski\(^{255}\) were used to transfer this design to light-inducible MBs. They connected a photoremovable quencher in
close proximity to a fluorophore of choice. Both modifications were applied on nucleobases. Like this, Dmochowski achieved a read-out for the uncaging kinetics of caged oligonucleotides. While he used Fluorescein and a collisional quencher (photocleavable Dabsyl), this approach could easily be adapted to alternative fluorophore-quencher-pairs.\textsuperscript{255}

![Figure 79: Within a DNA strand, two adjacent nucleobases were modified. Fluorescein was attached to one base and a dark quencher to the second one. The quencher is attached via a light-responsive moiety. Upon irradiation with 355 nm light, fluorescence can be observed.\textsuperscript{255}](image)

In a similar approach, Johansson et al. described a strategy for the labelling of proteins in a photoresponsive manner. The group applied a fluorophore of choice in direct neighbourhood of a photoremovable quencher. This construct was then coupled covalently to guanosine via its oxygen atom at position 6. Within a cell there are repair mechanisms to remove O-6-alkylations in a suicidal manner by transferring the moiety to a cysteine within the peptide sequence of the suicide enzyme. This protein is called \textit{O-6-alkyl guanine alkyl transferase (AGT)}. In 2004, this mechanism was used to specifically label proteins of interest with a number of different modifications. Therefore, the protein of interest had to be fused to an AGT derivative, the so-called \textit{SNAP tag}.\textsuperscript{256,257} So Johansson and his group could easily design different photoinducible probes for protein labelling to be used in high-resolution microscopy techniques such as PALM (photoactivated localisation microscopy).\textsuperscript{258} In this microscopy technique, only a small number of fluorescent molecules is supposed to lighten up by careful photoactivation. The activated fluorophore can then be distinguished from neighbouring inactive fluorophores, even though the resolution of the microscope is theoretically not sufficient.
There are several possibilities to adapt the design by JOHNSON et al. to light-inducible MBs. As a first approach, a design was supposed to be realised as shown in Figure 81. In an elaborate synthesis, an amino modified version of NPE should be attached to a nucleoside phosphoramidite for application in SPS. Afterwards the amino group could have been labelled with a dark quencher. This nucleotide could then be attached in an MB design next to the fluorophore within the stem sequence to allow for quenching of the fluorophore until the additional quencher is removed by light. Apart from the 14-step synthesis, which had been established by MAX BUFF during his PhD Thesis, this approach would have needed an orthogonal labelling strategy for the fluorophore and the additional quencher. This fact might again cause restriction in the fluorophore choice, because some dyes might not be available as necessary modifications.
Figure 81: Initial design plan based on the PhD Thesis by MAX BUFF. The nucleobase that is in direct
neighbourhood of the fluorophore needs to be modified with an amino modified o-nitrobenzyl derivative
as photocleavable element. This amino group can be modified post-synthetically with a dark quencher.
As a result, the MB can bind to its target without the emission of light. Only after uncaging and cleavage
of the NB-quencher moiety, fluorescence can be observed.

Besides, the bulky modification of the nucleobase might have a strong impact on the
thermodynamic stability of the MB stem, especially since studies have shown the influence
of one PPG to all neighbouring base pair stabilities.\textsuperscript{50,51} The effect could have completely
changed the secondary structure of the MB. Therefore, another design approach seemed
more advantageous. Here, a commercially available photocleavable (PC) linker was chosen
to connect a fluorophore of choice with the respective quencher. This approach will be
termed \textit{caged Q-dye MB} and is schematically drawn in Figure 82.

In this design, the fluorophore of choice is indirectly caged by an additional quencher that
can be removed photoresponsively. Importantly, in contrast to the two published designs by
HECKEL et al. and TAN et al., the caged Q-dye MB is able to bind to its target sequence even
before photoinduction. Therefore the fluorescence of target-bound MBs can directly be
observed after uncaging. Worth mentioning, the “common” dark quencher at the respective other end of the MB is still necessary to avoid false positive fluorescence read-outs after uncaging of unbound MBs.

As shortly mentioned above, a previously well optimised MB was used in this study for the detection and possible tracking of BR2.1 mRNA of *C. tentans* salivary gland cells. The loop-caged MB design needed to be directly compared with the caged Q-dye design.

For the synthesis of the loop-caged MB, NPP-modified dG phosphoramidite (dG$^{\text{NPP}}$) was first synthesised according to published procedures, schematically shown below.²⁵⁹

![Synthesis of dG$^{\text{NPP}}$ phosphoramidite](image)

**Figure 83:** Synthesis of the dG$^{\text{NPP}}$ phosphoramidite used in SPS for this study. In total, it is a six-step synthesis.²⁵⁹

To ensure the smallest possible number of NPP modifications used for the loop-caged MB, a test had to be performed. To be cost-efficient, three loop-caged test-MBs were synthesised as DNA-based MBs in contrast to the final MB that was supposed to be synthesised as 2′-O-methyl-RNA. It could be shown that seven NPP modifications showed the best results, meaning the highest on/off ratio compared to a non-caged control MB (positive control). The introduction of more than seven PPGs did not seem necessary. Due to good experiences in the past, we chose cyanine 3 (Cy3) as fluorophore ($\lambda_{\text{ex}} = 550 \text{ nm} / \lambda_{\text{em}} = 570 \text{ nm}$) and the Black Hole Quencher (BHQ) 2 as dark quencher (absorption range: 560-670 nm)
Figure 84: BR2.1 MB for investigation of mRNA from *Chironomus tentans*. The positions of NPP-modified guanosines are marked in green. Three different variants were tested. The secondary structure compared to the 2’-O-methyl RNA MB is slightly different due to possible G-U base pairing of RNA.

Figure 85: Results of loop-caged test MBs. All caged MBs show a low fluorescence signal in the absence of target. In the presence of target, solely MB1a with seven dG
NPP modifications show a satisfying background value of below 10% compared to full fluorescence of an unmodified positive control MB.

Regarding the new caged Q-dye MBs, the following dye combinations were chosen for this study. Worth mentioning, not all chosen dyes can be introduced directly via SPS. In contrast to the chosen dark quenchers, which are available as 5’-end phosphoramidite or 3’-end solid support, some of the dyes needed to be attached post-synthetically, in particular AttoTec dyes and TexasRed. The according coupling chemistry for each dye will be discussed in the following parts. For all MBs, 2’-O-methyl RNA is used.
Table 3: Overview of all MBs used for this study, the chosen names, and their dye composition.

<table>
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<tr>
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<th>3’-end quencher</th>
<th>5’-end dye</th>
<th>5’-end quencher</th>
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<td>Caged Q-dye</td>
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<td>MB1a</td>
<td>BHQ2</td>
<td>Cy3</td>
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<td>MB2</td>
<td>Dabcyl</td>
<td>Fluorescein</td>
<td>Dabcyl</td>
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<td>MB3</td>
<td>BHQ1</td>
<td>Fluorescein</td>
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<td>MB4</td>
<td>BHQ2</td>
<td>Cy3</td>
<td>BHQ2</td>
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<td>Atto550</td>
<td>BHQ2</td>
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<tr>
<td>MB6</td>
<td>BHQ2</td>
<td>TexasRed</td>
<td>BHQ2</td>
</tr>
<tr>
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<td>BBQ</td>
<td>Atto647N</td>
<td>BBQ</td>
</tr>
<tr>
<td>MB8</td>
<td>BBQ</td>
<td>Atto647</td>
<td>BBQ</td>
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<tr>
<td>MB9</td>
<td>BHQ3</td>
<td>Atto647N</td>
<td>BBQ</td>
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Regarding MB1a (dG\textsuperscript{NPP} loop-caged MB), only Cy3 and BHQ2 were chosen for this study due to the elaborate and time-consuming preparation, which did not seem appropriate for a control MB. This fluorophore quencher pair had been shown to work satisfyingly in previous studies by MIKAT et al.\textsuperscript{254}

Results for Caged Q-Fluorescein MBs (MB2 & MB3)

Even though Fluorescein has some certain drawbacks that limit its usage in single-molecule microscopy applications, as it has been mentioned previously, it is still one of the most popular and an easily available fluorescent dye in life science. Fluorescein can be introduced into an oligonucleotide via different ways. In this case, an internally introducible phosphoramidite is used (cf. Figure 86). The necessary tert-butylcarbonyl protecting groups are acid labile and are easily removed during final DMTr deprotection using 80% acetic acid for 25 min at room temperature.

Regarding MB applications, Fluorescein can be quenched by two different dark quenchers: Dabcyl, a widely used collisional dark quencher, and BHQ1, a sterically more demanding FRET quencher. Two designs will be tested, as it is schematically shown in Figure 86. MB2 bears the collisional quencher Dabcyl twice at both ends of the MB. Dabcyl has the advantage of being universal and its quenching efficiency is mostly independent from the fluorescence wavelength, in particular in a range of 350-500 nm emission wavelengths. Additionally, Dabcyl is the most cost-efficient choice. Due to the necessity of direct contact to the fluorophore, the quenching efficiency decreases drastically with distance. Due to that fact, the quenching was found not to be optimal, even if the quencher was attached at both ends of the MB.

Therefore, MB3 was designed with the FRET quencher BHQ1 at its 3’-end. To date, Black Hole Quenchers seem to be the most efficient dark quenchers that are commercially available. Nevertheless they are rather hydrophobic and sterically demanding.
Figure 86: Principle of caged Q-Fluorescein MBs (left). This design allows for target RNA (T) binding even before light-activation (+hν). The right side depicts the molecular structures of the photocleavable linkage (green) between Fluorescein (orange) and the respective dark quencher (Dabcyl, black) at the 5'-end of the MB. At the 3'-end two different dark quenchers were tested in this study, Dabcyl and BHQ1. All modifications were inserted as commercially available phosphoramidites.

Regarding the fluorescence results, both MBs perform very well. After 3 min illumination (250 mW, 365 nm, 500 pmol probe), both MBs are 95% uncaged without any visible side products.

The relative fluorescence increase is measured relatively to a non-caged positive control set as 100% in the presence of target. All fluorescence measurements were started after mixing and incubating the respective MB with its target RNA for 2 min to ensure complete binding. The results are depicted in Figure 87. The background fluorescence of MB2 and MB3 are rather low with 1.4±0.3% and 0.9±0.5%, respectively. The background fluorescence of MB3 seems slightly lower. It could be hypothesised that BHQ1 as FRET quencher might be more efficient than Dabcyl. After uncaging (+hν), but still without the target RNA, the background fluorescence of MB3 is again lower (4.0±0.8%) compared to MB2 (7.0±0.8%), most likely for the same reason. In the presence of target RNA, both caged MBs perform similarly around 2.0%, as it would be expected. The quencher that is adjacent to the fluorophore was chosen to be Dabcyl in both cases. After uncaging in the presence of target (approx. 1.5 eq.) both MBs regain almost full fluorescence (~95%).

In summary, the SNR is comparable after uncaging with an ON/OFF ratio of approx. 50:1 for both MBs, independently from their 3’-dark quencher. Nevertheless, the BHQ1-bearing MB3 performs slightly better when comparing the caged state in the absence of target with the full fluorescence (1:67 for MB2 and 1:107 for MB3, respectively).
Results for Caged Q-Cy3 MB (MB4)

The next dye-caged MB (MB4) was synthesised with an internal Cy3 phosphoramidite modification and two BHQ2 dark quenchers at both sides of the MB. Alternatively, there are also Sulfo-Cy3-NHS-esters available for post-synthetic amino group labelling. The common Cy3 derivative shows an increased hydrophobicity. For the application within an oligonucleotide with its negatively charged phosphate backbone, solubility was not an issue during most studies performed during this PhD Thesis. This hydrophilic Sulfo-Cy3 might become of interest for shorter MBs, if the hydrophobicity of the dark quencher becomes more prominent.

The uncaging efficiency of MB4 was comparable to the one measured for MB2 and MB3. After 2 min, almost no caged MB (150 pmol) was remaining after illumination with 250 mW at 365 nm. 150 pmol starting material of MB4 was used, as it has been determined by RP-HPLC (cf. Figure 89).

Figure 87: Performance of the two caged Q-Fluorescein MBs by \textit{in vitro} fluorescence. All fluorescence intensities are relative to the one of an unmodified Fluorescein-labelled MB. MB2 is labelled with Dabcyl as dark quencher at both ends, while MB3 contains BHQ1 at its 3’-end. The SNR of MB3 is approx. 1.5 times higher than the one of MB2.

Figure 88: Schematic view of caged Q-Cy3 MB4. The respective phosphoramidites for solid phase synthesis are shown as well. Cy3-phosphoramidite is shown in magenta, the photocleavable linker in green, and the quencher BHQ2 in full-black. In this case, the 3’-end contains the dark quencher BHQ2 as well.
Since the loop-caged MB1a was also Cy3- and BHQ2-labelled, a direct comparison between both MB designs was possible. Figure 90 depicts the relative fluorescence results. As for MB2 and MB3, a non-caged MB was used as positive control reflecting 100% fluorescence. The loop-caged MB1a showed a background fluorescence of 5.0±0.5%. Notably, in the caged Q-Cy3 MB4 design, the second quencher, being in very close proximity to the fluorophore, reduces the background fluorescence signal tremendously (0.6±0.3%). As expected, after uncaging, but still without the target RNA, both Molecular Beacons exhibited comparable fluorescence (approx. 4.0%), because the fully uncaged MBs are expected to be identical to the non-caged positive control. Importantly, MB1a still showed some increase in fluorescence upon addition of the target RNA (10.0±1.6%), while the fluorescence of MB4 remains very low after addition of the target RNA without uncaging. It can be reasoned that the 5'-dark quencher is more efficient than the 3'-dark quencher due to its proximity and most likely also due to additional contact quenching. Upon uncaging and addition of target RNA, the caged Q-Cy3 MB4 regains almost full fluorescence (99.5±1.7%). This is in contrast to the loop-caged MB1a with a maximal fluorescence value of 89.0±1.5%, most likely due to incomplete photocleavage of NPP-groups. As discussed previously, NPP groups tend to show irreversible side reaction at certain pH values.
Light-inducible Molecular Beacons

Figure 90: Comparison of the performance of the loop-caged MB1a and caged Q-Cy3 MB4 by in vitro fluorescence measurements. All fluorescence intensities are relative to the one of unmodified Cy3-labelled MB in the presence of target RNA. The SNR of the new caged Q-dye MB design is approx. 10 times higher compared to the former loop-caged MB. Besides, caged Q-dye MBs are able to restore maximal fluorescence after uncaging.

Apart from 2'-O-methyl-RNA-based MB, the Cy3-labelled MB1a and MB4 were also synthesised as DNA-based probes for preliminary experiments. Using DNase I, it can be nicely shown that the new caged Q-dye MB-design could prevent false positive results due to nuclease-caused digestion prior to uncaging.

When loop-caged, DNA-based MBs were incubated with DNase I, the fluorescence increases rapidly, since fluorophore and quencher are easily separated, once the stem structure is destroyed. Maximal fluorescence, based on the equivalent amount of Cy3, is achieved after 3-4 min. In contrast, the covalent connection between fluorophore, PC linker, and second quencher was not cleaved by DNase I. Fluorescence was constant without any increase for up to approx. 10 min.

Notably, these findings are only true in the case of a fully caged MB. After uncaging, the MB corresponds to a non-caged MB and can therefore be digested again. Nevertheless, usually only a small fraction of MBs are uncaged to gain the best signal-to-noise ratio. Besides, the usage of nuclease-resistant derivatives such as 2'-O-methyl-RNA helps to delay nuclease-mediated degradation massively.
Figure 91: A DNA-based loop-caged Molecular Beacon (MB1a, red) is rapidly degraded by DNase I. As a result, the fluorescent signal increases even in the absence of target and without light-activation. A caged DNA-based Q-Cy3 MB4 does not show an increase in fluorescence (blue). False positive results are reduced. The linkage between fluorophore and quencher of MB4 is not cleaved by nucleases. The fluorescence is based relatively to the fluorescence of an equal amount of Cy3.

Generally, these findings impressively show that the newly introduced caged Q-dye MB design might be superior in terms of SNR and stability. The new design for caged Molecular Beacons greatly outperforms previous designs. In general, it is possible to adapt this design to any fluorescent dye and its respective quencher, even though there are some drawbacks, as discussed in the following subchapters. All these characteristics might make it possible to visualise and track RNA molecules upon uncaging in real-time and in a well-resolved manner.

Results for Caged Q-Atto550 MB (MB5)

As a very promising alternative to Cy3, Atto550 shows a 5.5-times higher quantum yield (0.80 vs. 0.15), even though the extinction coefficient is well comparable (120,000 M⁻¹cm⁻¹ vs. 135,000 M⁻¹cm⁻¹ at the respective λ_max). These photochemical data make it suitable for real-time single-molecule imaging. Apart from that, it is much more stable under both uncaging (365 nm, 300 mW) and excitation (565 nm, 90 mW) conditions. Figure 92 depicts the fluorescence decrease during illumination of a 100 µM dye solution for up to four days.

Figure 92: Fluorescence decrease of Cy3 and Atto550 during illumination with 365 nm (300 mW, left) and 565 nm (88 mW, right). Atto550 is photochemically more stable and might therefore be more suitable for real-time tracking experiments.
Due to its photochemical advantages, Atto550 was chosen for the design of the caged Q-Atto550 MB5. MB5 was designed in a similar manner to MB4. Worth mentioning, Atto550 is not available as phosphoramidite and must therefore be attached post-synthetically to the oligonucleotide of choice. In this case, an internal amino modifier was used.

After synthesis, deprotection, and initial purification, the MB5 precursor was labelled with Atto550-NHS active ester. The structure of the fluorophore has not yet been disclosed by the company to date. The labelling yields were absolutely satisfying with up to 90%, when 1.5 equivalents of the NHS ester were used compared to 1 equivalent of MB5 amino modified precursor. The yield was determined according to HPLC peak areas during post-labelling purification.

The uncaging procedure proceeds without side reactions. After illumination of 150 pmol of the caged Q-Atto550 MB5 for 2 min (365 nm, 250 mW), no caged compound is left. Further fluorescence measurements revealed that the SNR for MB5 was similar to the previously introduced dye-caged MBs.

The caged Q-Atto550 MB5 performs comparable to the previously shown caged Q-Cy3 MB4. Notably, in this case the Atto550 dye was attached via a (CH$_2$)$_4$-linker and showed a slightly higher background signal (1.1±0.4%). This finding can most likely be explained by the higher distance between fluorophore and quencher, making FRET quenching less effective.
General Part

Figure 94: Fluorescence in vitro results of caged Q-Atto550 MB5. The fluorescence increases 85-times after uncaging after addition of target RNA. If target RNA is added prior to uncaging, the fluorescence increase is 54-times compared to the caged background fluorescence.

Figure 95: Uncaging of caged Q-Atto550 followed by RP-HPLC analysis. 150 pmol MB5 were used per measurement. After approx. 90 s, no caged compound is left. During uncaging, no side reaction can be observed.

Results for Caged Q-TexasRed MB (MB6)

An additional dye-caged MB containing TexasRed and two BHQ2 labels (MB6) was synthesised to examine the influence of the linker length. TexasRed has the shortest linker length available for fluorescent dyes and its fluorescence properties are comparable to Cy3 ($\lambda_{ex} = 586$ nm / $\lambda_{em} = 605$ nm / $\epsilon = 85,000$ M$^{-1}$cm$^{-1}$ / $\phi = 0.61$). A shorter linker was thought to additionally improve SNR results of the MB due to higher quenching efficiency. TexasRed is available as sulfonyl chloride and can be attached to amino groups.

Figure 96: Molecular structure of TexasRed. The fluorophore belongs to the group of rhodamine-based dyes. Its excitation maximum is at 586 nm with an emission maximum at 605 nm. The dye is commercially available as highly reactive sulfonyl chloride, which is directly attached to the fluorophore.
Due to the massively increased reactivity compared to NHS esters, sulfonyl chlorides could be attached to both aliphatic and exocyclic amino groups with the latter ones being present in high excess within an oligonucleotide. Therefore the labelling procedure turned out to be much more elaborate, as it had to be conducted on solid phase with protected exocyclic amino groups at the nucleobases. For this reason, an Fmoc-protected amino linker was used. This protecting group can be removed under very mild basic conditions that do not cleave off the protecting groups of the nucleobases.\textsuperscript{260,261} To avoid the alkylation of amino groups by acrylcyanide, the cyanoethyl protecting group of the phosphoramidite itself needed to be removed in an initial reaction step. The TexasRed labelling was performed on solid support manually. As the instability of related rhodamine dyes such as TAMRA against ammonia or methylamine is known, the labelled solid support was aliquoted and partly deprotected using ammonia at room temperature for 18 h and partly using the so-called TAMRA cocktail (t-butylamine/water (1:3, (v/v)), 6 h, 60 °C). Both deprotection conditions showed similar results with the ammonia deprotection even slightly better ones. Nevertheless, the labelling reaction yield was generally low (≤6\%) and the results did not show any improvement compared to Cy3- or Atto550-labelled caged Q-dye MBs. The SNRs were almost the same with ON/OFF ratios of 100:1 and 70:1 in the absence or presence of target, respectively.

\begin{figure}[h]
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\caption{Fluorescence \textit{in vitro} results of caged Q-TexasRed MB6. The fluorescence increases 105-times after uncaging after addition of target RNA. If target RNA is added prior to uncaging, the fluorescence increase is 68-times. TexasRed can be attached to the MB with the shortest possible linker.}
\end{figure}

\section*{Results for Caged Q-Atto647N MBs (MB7 & MB8)}

According to experiments of our cooperation partners, Atto647N seemed to be the most stable and best suitable fluorescent dye for single-molecule tracking ($\lambda_{ex} = 644 \text{ nm}$/ $\lambda_{em} = 669 \text{ nm}$/ $\varepsilon = 150,000 \text{ M}^{-1}\text{cm}^{-1}$/ $\phi = 0.65$). Besides, red-shifted dyes with an excitation and emission maximum above 600 nm are of special interest, since light of lower energy tends to penetrate most tissues more easily and with less cell destruction.

In this study, Atto647N was combined with two BBQ quenchers (Black Berry Quencher) on a 2′-O-methyl-RNA MB termed MB7. The fluorophore itself was purchased as active ester and attached to the MB7 precursor via the internal amino modifier that had already been introduced for the Atto550-caged MB5 (cf. Figure 98). Worth mentioning, the structure of Atto647N has not yet been disclosed by AttoTec to date, but NMR-based studies suggest a rhodamine-type fluorophore\textsuperscript{262}. 

Figure 98: Schematic view of caged Q-Atto647N MB7. The molecular structure of the 5'-end is shown as well as the structure of the 3'-BBQ solid support. Atto647N-NHS ester is labelled post-synthetically to a (CH₂)₄-amino modifier.

Unfortunately, the fluorescence results did not show the same good uncaging behaviour, as it had been achieved for the other caged Q-dye MBs. Instead, a massive fluorescent background was observed, even prior to uncaging and in the absence of target. Despite the fluorescence results, HPLC analysis showed complete uncaging after 90 s of illumination (365 nm, 250 mW) of 150 pmol starting material, which is comparable to previously investigated MBs.

First, it was assumed that the positively charged Atto647N moiety might interfere with the negatively charged phosphate backbone of the oligonucleotide. Therefore, the MB7 precursor was labelled with the Atto647N derivative called Atto647. This dye is not charged, but has the disadvantage of being base labile, so that even the labelling process could not be conducted without impaired yields due to base-catalysed degradation of the dye. Even the change of the labelling pH from commonly buffered 8.5 to 8.0 did not improve yields significantly. The uncharged Atto647-caged MB8 showed the same high background fluorescence. The SNR was low and the MB fluorescence did not seem to be quenched efficiently.
The linker length as a possible explanation for the observed results of MB7 and MB8 was excluded due to the prior study with the caged Q-TexasRed MB6. This dye was attached to the MB with the shortest possible linker length available, but SNR results did not improve. Another possible explanation might be the hydrophobicity of red-shifted dark quenchers. The lower the photoenergy that has to be quenched, the bigger the chromophoric π-system causing decreasing water solubility. It could be hypothesised that due to stacking of the two hydrophobic BBQ dyes, they did not quench the fluorescence anymore. Stacking could be proven due to a bathochromic shift of the absorption spectra compared to a diluted solution of BBQ.

To possibly disturb the stacking of identical aromatic molecules, another FRET quencher called BHQ3 was chosen to be attached to the 3’-end (MB9). Importantly, BHQ3 is known to be extremely base-labile and therefore difficult to handle during SPS cleavage procedures and further amino labelling reactions. After SPS the deprotection was performed under Ultramild® conditions. Therefore, easily removable protecting groups for the nucleobases had been used for SPS such as acetyl-protected 2’O-methyl-C, PAC-protected (phenoxyacetyl-) 2’O-methyl-A, and 4-isopropylphenoxy acetyl (iPrPAC) 2’-O-methyl-G phosphoramidites. These groups can be removed using ammonia at room temperature within only 2 h. Even though this final SPS yield of the precursor MB8 was 2-times lower compared to other syntheses (35% (7.0 nmol)) after initial purification, the correct product could be isolated. The subsequent Atto647N-NHS ester labelling was performed at pH 8.0 instead of pH 8.5, as it had already been done in the case of the base-labile Atto647-labelling. Additionally, reaction time was decreased from regular 90 min to 30 min. Unfortunately, after labelling it was not possible to isolate the correctly labelled MB9. The resulting HPLC chromatogram suggested several degradation side reactions of the oligonucleotide, but neither pure nor successfully labelled MB9 could be purified. As a consequence, BHQ3 should be avoided in SPS applications that require basic work-up, as BHQ3 seems to degrade over time, depending on the base and the solvent. While degradation due to aqueous ammonia seemed acceptable, it was impossible to resolve undegraded product after NHS-ester labelling conditions in this case. Instead, thiol-
modifications could be introduced into the oligonucleotide and labelled post-synthetically with a maleimide-modified dye, as this labelling procedure is usually performed at neutral pH conditions.

**In vivo Application of Caged Q-Dye Molecular Bacons**

According to preliminary fluorescence measurements, the most suitable caged Q-dye MBs were chosen for further *in vivo* experiments in salivary gland cells of *C. tentans*. These experiments were performed in collaboration with the group of Prof. Ulrich Kubitscheck from the University of Bonn and his PhD student Tim Kaminski.

Table 4 shows an overview of the MBs that were chosen for this study.

<table>
<thead>
<tr>
<th>Quencher (3’/ 5’-end)</th>
<th>Fluorophore (5’-end)</th>
<th>$\lambda_{ex}$ / $\lambda_{em}$ / nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loop-caged MB1a</td>
<td>BHQ2 / -</td>
<td>Cy3</td>
</tr>
<tr>
<td>Cy3-caged MB4</td>
<td>BHQ2 / BHQ2</td>
<td>Cy3</td>
</tr>
<tr>
<td>Atto550-caged MB5</td>
<td>BHQ2 / BHQ2</td>
<td>Atto550</td>
</tr>
</tbody>
</table>

MB1a and MB4 were included into the study to allow for direct comparison between the loop-caged and caged Q-dye MB design, while MB5 was labelled with the superior fluorophore Atto550, which might be suitable for future real-time mRNA tracking.

The chosen MBs were applied to salivary gland cells of *C. tentans*. In a first experiment, the activation efficiency of Cy3-labelled MB1a and MB4 were compared. Both MBs were co-injected into *C. tentans* salivary gland cell nuclei with the Atto550-caged MB5 as internal reference. The hybridisation time for the Molecular Beacons was expected in the order of 2-20 s. After this time period, the visualisation was started. It was possible to determine the activation efficiency of the two MB designs due to the fact that Atto550 and Cy3 display different spectral properties. Like this it was possible to compare both Cy3-labelled designs to the caged Q-Atto550 MB5 and subsequently the two Cy3 species with each other indirectly.

First, separate reference spectra of Atto550 and Cy3-labelled MBs were determined. To this end, either MB4 or MB5 were microinjected into nuclei of *C. tentans* salivary gland cells, as it is described in literature. In contrast to *in vitro* illumination experiments, which are described in the materials chapter, the MBs had to be uncaged *in vivo* under the microscope in this case. Therefore, the gland cells were illuminated with the high pressure mercury arc lamp of the fluorescence microscope using a DAPI filter set for 10 s. This time was found to be the optimal illumination time. As a result, spectrally resolved images of the gland cell nuclei were acquired. Images showing the brightest five spectral channels are shown below in Figure 100.
Light-inducible Molecular Beacons

Figure 100: Detection of caged Q-Cy3- and caged Q-Atto550 Molecular Beacons MB4 and MB5 that bind to the BR2.1 mRNPs inside of C. tentans salivary gland cell nuclei. The panels show intranuclear regions containing the BR2.1 transcription sites (marked with arrow heads) after illumination for 10 s with UV light. It is obvious that Atto550 and Cy3 have slightly differing emission maxima.

In each image the region of the MB-mediated fluorescently labelled BR transcription site was selected and the average intensity of that region was measured. The resulting spectra were measured in several different nuclei and averaged, yielding the required reference spectra.

In the next step, a mixture of either MB1a and MB5 or MB4 and MB5 was co-injected in a molar ratio of 1:1 into nuclei of salivary gland cells as before. After uncaging, spectral images of the BR transcription sites were acquired. Again, the mean spectral intensity of the BR transcription site was determined and compared for each nucleus.

The relative contributions of Cy3 and Atto550 were determined in ten different nuclei for each beacon combination and averaged. Still, it had to be considered that the excitation and detection probabilities of both dyes in the instrument were not identical. Additionally, the different maximal molar extinction coefficients, the different excitation efficiency at 543 nm, and the different fluorescence quantum yields had to be taken into account and correlated to each other mathematically. All this was conducted by the group of PROF. ULRICH KUBITSCHHECK. The result is depicted in Figure 101.

Figure 101: The amounts of MB1a and MB4 in living C. tentans cell nuclei upon uncaging were determined relative to that of MB5, as described above. Thus, the signal intensity ratio of caged Q-Atto550 MB5 after uncaging was set to 1 by definition, and the signal increase of MB1a and MB4, respectively, was determined in relation to that. The figure shows that the signal increase of the caged Q-dye design of MB4 compared to the loop-caged MB1a was up to 5-fold.
In the next step, the performance of the loop-caged design and the new caged Q-dye design was evaluated in vivo. To do so, loop-caged MB1a and caged Q-Cy3 MB4 were microinjected into *C. tentans* salivary gland cell nuclei. The cells were imaged using confocal laser scanning microscopy before and after photoactivation. The results are depicted in Figure 102.

![MB1a and MB4 in vivo comparison](image)

**Figure 102:** Comparison of the loop-caged and the caged Q-dye beacons, MB1a and MB4, in vivo. Both beacons were labelled with Cy3 and were microinjected into *C. tentans* salivary gland cell nuclei. After photoactivation by UV light, MB4 exhibited a significantly stronger fluorescence signal than MB1a. The BR2 transcription sites are indicated by arrows. Scale bar: 10 mm, the data acquisition parameters were identical in all cases.

Before photoactivation (-hv), MB4 showed a significantly lower background fluorescence than MB1a. Photoactivation (+hv) was conducted by illumination with UV light of the microscope's mercury high pressure lamp for 10 seconds, as it has been described above. With both beacons, the BR2 transcription sites became distinctly labelled. The BR2 transcription site is characterised by its doughnut-like structure and was expected to be the region with maximal concentration of target RNA.

Caged Q-Cy3 MB4 resulted in a strikingly stronger fluorescence signal than MB1a. It should be noted that the fluorescence signal increase after photoactivation of the BR2 transcription site was in the range of 80-fold for MB4, but only 10-fold for the loop-caged MB1a. Nevertheless, such high contrast ratios are difficult to measure and in the present case the signal obtained with MB4 before irradiation was almost negligible. Therefore, SNR values like this should be handled with care. However, the images and these numbers show that the performance of MB4 over MB1a in a cellular environment is at least as good as in the previous *in vitro* experiments (63-fold for MB4 versus 9-fold for MB1a).

From the in vivo experiments it can be concluded that the new caged Q-dye design for a caged Molecular Beacon greatly outperforms previous designs. The caged Q-dye design is ideal for bulk mobility measurements of specific RNAs after uncaging. Beyond that, this design will allow for completely new experiments in visualising and tracking of mRNA molecules upon uncaging at defined intracellular loci such as transcription sites or nuclear pore complexes.
Caged Quantum Dot-labelled Molecular Beacons

During this thesis, another approach of light-inducible Molecular Beacons was tested. Especially the synthetic part of this study was mostly conducted during the Bachelor Thesis of SVENJA R. BIRON. In this study, inorganic quantum dots (QDs) replaced the common organic dyes at the end of an MB. Inorganic QDs have already been introduced briefly in the introduction chapter. They have become popular during the last years due to their superior photochemical properties. They show a broader spectral range compared to organic fluorophores and are reliably excitable at low light intensities. Additionally, they are less prone to photobleaching, a feature that would make them suitable to microscopy techniques with high excitation light intensities. The fact that QDs can be excited by two-photon excitation is an additional advantage for in vivo applications, because fluorescence can be excited in a small region within the focal plane of the microscope. As a result, no background fluorescence lowers the SNR and the red-shifted excitation wavelength is well known to be biocompatible. The surface of QDs can easily be functionalised by thiols, as the QDs themselves or their shell consist of sulphides for the formation of covalent dithiol bonds. This surface functionalisation can be used to increase hydrophilicity and for labelling of oligonucleotides and proteins. It must be noted, though, that this surface functionalisation might influence optical properties negatively.

Despite all advantages of QDs, their drawbacks must be mentioned as well. It has not been proven explicitly whether QDs are cytotoxic or not. There have been studies claiming the biocompatibility of QDs, e.g. by CHEN AND GERION in 2004. In contrast, other studies have shown that QDs might damage tissue, because toxic components such as cadmium ions might exit the QD and diffuse into the cell as a result of oxidation processes. Regarding possible applications, it is known that QDs do not show constant fluorescence, but rather blink due to defects within the crystal lattice that interfere with fluorescence emission. Even though blinking fluorophores cannot be used for real-time tracking, they are interesting for other microscopy techniques such as dSTORM (abbrev.: direct stochastic optical reconstruction microscopy). dSTORM commonly uses organic dyes that are forced to blink by oxidising and reducing buffer systems. Redox reactions make dyes stochastically enter the dark triplet state due to intersystem crossing causing blinking when the dye drops back into the ground state. Like this, the exact position of one single dye can be calculated with the help of the point spread function (PSF) resulting in a single-molecule resolution that is lower than the microscope would usually allow.
In addition, an interesting study by Bruce E. Cohen in 2008 introduced a possibility to make QDs light-inducible.\textsuperscript{278} Generally, QDs can easily be quenched by dark quenchers due to the FRET effect comparably to common organic dyes. In addition, QDs are also quenched by organic aromatic molecules due to electron exchange. Electrons can be exchanged either between the QD conduction band and the LUMO of the quencher or between the HOMO of the quencher and the QD valence band. Both cases prevent the excited QD from relaxing back to the ground state and emitting fluorescence, as it is shown in Figure 104.\textsuperscript{271}

For this reason, it is possible to make QDs light-inducible by attaching photocleavable moieties such as o-nitrobenzyl groups to the QD.
This principle was reproduced and expanded to different PPGs and also applied to MBs. It was possible to modify commercially available QDs (Lumidot™ CdSe/ZnS by Sigma Aldrich) with thiol-modified PPGs. SVENJA R. BIRON also managed to test different linker structures of NPE- and NPP-caged QDs during her Bachelor Thesis. Depending on the leaving group, the SNR was found to be between 1:100 and 1:50. As one would expect, phosphate was found to be the best leaving group with the highest SNR. The QDs were illuminated by light (365 nm of 250 mW) for 2-5 min.

When NPE was used as PPG, the SNR of the phosphodiester linkage was found to be 1:100 and 1:80 in the case of NPP. Carboxylic acid ester linkages showed an SNR of 1:50, if NPP was used as PPG. The fact that NPE performed slightly better can be explained by the known irreversible side reactions, which have been mentioned for NPP previously.

Worth mentioning, the 100% fluorescence value was determined by unmodified QDs. One could discuss that after uncaging the remaining alkyl group might impair fluorescence, so that the 100% fluorescence is not regained. However, if the respective alkyl groups were attached to the QDs, the results hardly changed. It could be hypothesised that the concentration of aromatic systems within solution is high enough even after photocleavage – to quench fluorescence, even though there is no covalent connection. Incomplete uncaging could be excluded, because longer illumination times did not improve the results. Sometimes the fluorescence even decreased, most likely due to photobleaching (data not shown).

Later on, it was also possible to attach pH – a very promising multi-photon cage that was heavily under study for DNA-based applications by RODRIGUES et al. Unfortunately, this cage did not result in sufficient quenching (SNR 1:9). Also the coumarin derivative DEACM (diethylaminocoumarin) showed a rather low SNR (1:4) – most likely due to its own fluorescence. Both PPGs were attached to the QD via a carbamate containing linkage that is also known to be a good leaving group due to the release of CO₂ after photocleavage. However, the synthesis of carbamate linkages is more elaborate due to the necessary usage of phosgene.
The most promising caged QD (NPE phosphodiester linker) was then attached to the thiol-modified BR2.1 MB. As discussed before, this MB had been well studied for a variety of different organic fluorophores. This fact makes it possible to compare and rate the results of a QD-labelled MB.

**Figure 106**: Schematic design of a caged quantum dot MB. The QD is non-fluorescent in the presence of aromatic moieties. Once, these moieties such as NB derivatives have been removed, e.g. by light, the QD is able to fluoresce in the presence of target, which opens up the MB.

When the BR2.1 target RNA was added to the MB, a fluorescence increase could be observed after illumination. Nevertheless, the SNR of this caged QD MB was inferior to the above introduced caged Q-dye MBs. The OFF/ON ratio before and after uncaging was well comparable to the preliminary data of caged QDs discussed above. However, the background fluorescence after uncaging in the absence of target is very high (OFF/ON ratio 1:10). This is most likely due to incomplete fluorescence quenching by BHQ2 either due to the long distance or due to the extreme difference in size.

**Figure 107**: Fluorescence results of a QD-labelled BR2.1 MB. The QD has been caged by NPE via a phosphate bond. The background fluorescence of the uncaged QD (red and green) is well comparable with the preliminary data of caged QDs alone. Nevertheless, after uncaging, in the absence of target, the background is rather high due to incomplete quenching of the QD by the dark quencher BHQ2 (yellow). Besides, 100% fluorescence cannot be regained (blue).

Apart from that, it was not possible to purify and characterise the caged QD MB reliably. It could not be excluded that more than one MB was attached to the caged QD. Neither RP-HPLC nor SEC-HPLC showed well-separable signals that could have been further analysed. For this reason, the caged QD approach was not further executed, as *in vivo* applications...
Light-inducible Molecular Beacons

need reliably purified probes to exclude cytotoxicity and intracellular side reactions as much as possible.

**Summary**

In summary, this project successfully developed a new architecture for light-inducible Molecular Beacons that can even outperform previous designs. In this project, commercially available fluorophores were indirectly caged by applying a photoremovable additional quencher in close proximity. Like this, the caged Q-dye MB can bind to its target RNA without fluorescence to occur, until photoinduction at sites of interest has happened. This approach can theoretically be applied to a wide variety of commercially available fluorophores, independently from their labelling strategy. Nevertheless it was not possible to use red-shifted fluorescent dyes in the present study due to stacking of the respective dark quenchers making them quenching-incompetent.

Selected fluorophores showed a superior signal-to-noise ratio up to 180:1 for caged Q-Cy3 MBs. Additionally, the design prevents false positive results due to nuclease-mediated degradation of the MB, as long as the MB stays in its non-induced state.

Two dye-caged MBs were selected for in vivo applications in salivary gland cells of *C. tentans*. These cells are of special interest due to the presence of polytene chromosomes, so-called Balbiani Rings that produce a high copy number of certain mRNAs and show very distinct transcription sites within the nucleus. The study shows that the background fluorescence is negligible even within a cell and that the photoinduction works well and locally restricted, again with superior SNR values.

As a result, this design could allow for visualising and tracking mRNA molecules upon uncaging at defined intracellular loci such as, for instance transcription sites, nuclear speckles or nuclear pore complexes.

It was also possible to label MBs with caged quantum dots that might be interesting due to their superior photochemical properties. Nevertheless, the SNR of caged QD MBs was not as good as the comparable caged Q-dye MB design using organic fluorophores, even though caged QDs in general worked well.
Future Prospects

It could be shown that single-molecule experiments for this MB design are still lacking, even though the superior SNR of the new caged Q-dye MB design would make it possible. It would still be desirable to apply this MB design to more photostable fluorescent dyes, which fluoresce in a red-shifted wavelength range. During this PhD Thesis, the issue of stacking hydrophobic BBQ dyes was not further investigated. Instead, alternative ways were hypothesised and planned to overcome the necessity of two hydrophobic quenchers in close proximity.

One promising opportunity would be the synthesis of directly caged cyanine 7 (Cy7) derivatives instead of using indirectly caged commercial dyes, as it was shown above. For this purpose, the so-called quinone-Cy7 (QCy7) might be a good option to test. QCy7 was first used in 2011 by SHABAT et al. as a general sensor dye.280,281 The QCy7 structure is based on a quinone system incorporated into the Cy7-π-system. In its hydroquinone state, the Cy7-structure with its characteristic push-pull-system is disturbed and no electron flow is possible.

Figure 108: Basic principle of QCy7 dyes. In its protonated state, the push-pull mechanism of the fluorophore is disrupted and no fluorescence can be observed. Deprotonation and formation of the quinone form regain the push-pull-mechanism and fluorescence can be observed. The exact excitation and emission values depend on the push-pull moieties, but generally, the Cy7 fluorescence is red-shifted at around 750 nm.

The hydroquinone structure can be forced by applying a protecting group that can be removed by its respective trigger signal. In literature, a number of protecting groups have already been applied for the usage as enzyme assay (galactosidase-, nitroreductase-dependent assay) or an oxidation-sensitive fluorescent read-out based on QCy7.281
Figure 109: QCy7-based probes for the detection of β-galactosidase activity. Upon enzyme-mediated removal of galactose, the fluorescence of the QCy7 dye becomes active again.\textsuperscript{281}

For the application in real-time mRNA tracking, a photocleavable approach would be the system of choice. The two students \textsc{Juliana Ersch} and \textsc{Alexandra Gresika} already succeeded in applying NPE to several push-pull systems to obtain a variety of caged QCy7 derivatives during their Bachelor and Master Theses, respectively.

Figure 110: a) Principle mechanism of caged QCy7 dyes. In this case, NPE is used as photolabile protecting group. Only after irradiation and removal of NPE, the push-pull mechanism is restored allowing fluorescence activity. b) Possible push-pull residues.

In theory, the strength of this system lies in the potentially fast synthesis. Preliminary studies have shown, though, that the purification of these dyes is rather elaborate and time
consuming due to its charged and hydrophilic nature. Besides, ALEXANDRA GRESIKA could show in her Master Thesis that some of the possible QCy7 derivatives are not stable under aqueous conditions making them unsuitable for biological *in vivo* applications.

*Figure 111: Synthesis of QCy7 with sulfo-indoles as push-pull pairs.*
Local miRNA Maturation

Visualisation of Local miRNA Maturation

In 2006, SCHRATT et al. identified a number of miRNAs including microRNA-134 and its precursor as dendritically localised. Dendritical levels of the pre-miRNAs could be proven by LNA-based FISH (fluorescence in situ hybridisation) experiments. In 2013, the group published a new study to further identify the structural elements that are necessary for specific localisation. For this reason, they labelled in vitro transcribed pre-miRNA-134 unspecifically with Cy3. Like this, the exact localisation of pre-miRNA-134 could be visualised. They could also show that pre-miRNA-150 that had previously been shown to be exclusively somatically localised was not transported to the dendrites. Even an artificial pre-miRNA that contained the stem structure of pre-miRNA-134 and the loop region of pre-miRNA-150 was analysed regarding its cellular localisation within neurons compared to the native pre-miRNA-134 itself. When this Cy3-labelled "hybrid" pre-miRNA-134L150 (pre-miRNA-134 stem combined with pre-miRNA-150 loop sequence) was transfected into hippocampal rat neurons, the signal was exclusively found within the cell body. In contrast, the converse hybrid pre-miRNA-150L134, which contained the loop sequence of pre-miRNA-134, fully restored dendritic localisation. It could be concluded that specific RNA-binding proteins (RBPs) might be responsible for the transport. Using pull-down experiments followed by MS analysis of the respective co-precipitated protein, they identified the DEAH box helicase DHX36. Interestingly, DHX36 was not found to bind other dendritically localised pre-miRNAs. For non-neuronal cells, it has been shown that DHX36 resolves secondary structures in the nucleus and seems to have an important role in the regulation of RNA metabolism. Its role in neurons was not understood at all until then.

Figure 112: The pre-miRNA-134 terminal loop is necessary and sufficient for dendritic targeting. Part A shows the sequences of in vitro transcribed pre-miRNA-134 (blue) and pre-miRNA-150 (grey). Figure part B depicts microscopy images of hippocampal neurons transfected with either of the Cy3-labelled pre-miRNAs. Arrows indicate fluorescence signal that is present in a punctate structure. These structures are solely present in the soma in the case of pre-miRNA-150, but can also be found in dendrites for pre-miRNA-134.
SCHRATT and his group chose pre-miRNA-134, because its role in dendrites has been well understood. In 2006, it had been shown that the expression of miRNA-134 is restricted to the brain and that the levels of mature miRNA-134 reach their maximum during synaptic maturation (post-natal day 13 (P13) in rat). SCHRATT and his group conducted in situ hybridisation experiments and overexpressed pre-miRNA in neuronal cells to show that the presence of pre-miRNA-134 or mature miRNA-134, respectively, leads to shrinkage of dendritic spines.\textsuperscript{163} As previously outlined, the size of dendritic spines reflects the ability of a dendrite to receive signal input and correlates to the strength of a neuronal network.\textsuperscript{288–290} These findings suggest not only the presence of certain specifically dendritically localised pre-miRNAs. Furthermore it can be hypothesised that these pre-miRNAs might be locally processed to mature miRNA upon certain signal input. After targeted transport, the final maturation of the pre-miRNA is most likely controlled in an activity-dependent manner.

To further investigate these hypotheses, we decided to work on miRNA-181 and its precursor (specifically on the isoform pre-miRNA-181a1) in rat hippocampal neurons. This specific miRNA was chosen for its importance and its diverse role within the central nervous system. MiRNA-181a was shown to bind to a highly conserved miRNA-181a binding site within the GluA2-mRNA, encoding for one of the most important subunits of AMPARs (cf. introductory chapter). Changes in miRNA-181a expression levels in primary neurons confirmed its role as negative post-transcriptional regulator of GluA2 expression. Besides, miRNA-181a overexpression caused GluA2 surface reduction and reduced miniature excitatory post-synaptic current (mEPSC) frequencies in hippocampal neurons. All these findings suggest that miRNA-181 is an important regulator for synaptic function. Moreover, SCHRATT et al. could show in mouse experiments that miRNA-181a expression was induced by a variety of organic molecules such as dopamine, cocaine, and amphetamines.\textsuperscript{291}

Unpublished data by SCHUMAN et al. suggest an additional role in regulating the kinase CaMKII. Like this, miRNA-181 would have a massive impact within cellular signal transduction.

Regarding its general role within the human organism, miRNA-181 seems to be important in a number of T-cell dependent immune responses. Increased expression of miRNA-181a in mature T-cells increases sensitivity to peptide antigens, while down regulation of miRNA-181a in immature T-cells leads to reduced susceptibility.\textsuperscript{292} Regarding its role in cancer, it has been shown that overexpression of miRNA-181 can be correlated with the development of neuroblastoma or chronic lymphocytic leukemia.\textsuperscript{293,294} In contrast, down regulation of miRNA-181a may lead to brain cancers such as glioma or glioblastomas – two very life-threatening tumours with rather poor survival prognosis.\textsuperscript{295,296}

**Probe Design and Primary in vitro Experiments**

To investigate pre-miRNA-181 maturation in neurons, the aim was the design of a probe that changes its fluorescence upon Dicer-mediated processing to the mature miRNA-181. A similar probe design has already been published in 2006 and 2008 by ARENZ et al. In this design, a fluorophore and a quencher were covalently attached to the 3’- and 5’-end, respectively, of a pre-miRNA of choice. The architecture is closely related to the basic design of a Molecular Beacon. In contrast to native pre-miRNAs, ARENZ’ design did not contain a 2-nucleotide overhang at its 3’-end, as this would lead to a higher distance between
fluorophore and quencher and impair FRET efficiency.\textsuperscript{297,298} Worth mentioning, the 2-nucleotide overhang is important for specific Dicer cleavage. Studies have shown that Dicer is not able to find its unique cleavage site, if there is no overhang present at a certain double-stranded RNA. Unspecifically cleaved, non-native products would be produced. Nevertheless, if no overhang is present, Dicer is able to digest a double strand twice, producing an RNase-type-III-like 2-nt-overhang at one or both sides.\textsuperscript{299} Therefore, ARENZ' blunt end design is not suitable for \textit{in vivo} applications that are supposed to reflect the native states. ARENZ and his group were not interested in the native cleavage product, but rather in Dicer activity or inhibition upon ligand binding. Like this, Dicer-inhibiting ligands such as certain peptides mimicking structural parts of Dicer could be identified, since there was no fluorescence increase. It must be noted that an optimal inhibiting ligand should not inhibit Dicer itself, but rather prevent Dicer from binding to the pre-miRNA and its maturation. A global inhibition of Dicer is most likely lethal for the organism in early developmental stages.\textsuperscript{300} ARENZ et al. also tested RNA binders such as kanamycin and a number of structurally related aminoglycosides.\textsuperscript{301} While kanamycin showed hardly any effect, some of the aminoglycosides were able to inhibit several tested pre-miRNAs to up to 100\%.\textsuperscript{298,302}

A design for the investigation of specific pre-miRNA maturation needs the characteristic 2-nt-overhang at the 3'-end of the probe. In another approach in 2013, GENG et al. designed a QD-based probe that can bind to complementary native pre-miRNAs. Like this, no pre-miRNA derivatives need to be delivered to the cell with their modifications potentially interfering with Dicer activity. The basic function of this assay is depicted in Figure 114. An RNA probe was labelled with a QD and a gold nanoparticle (Au-NP) as quencher on its two terminal sites. The sequence of the RNA probe was chosen to be complementary to a pre-miRNA of interest – to be specific at the region of intended Dicer cleavage. The FRET effect between the QD and the Au-NP quenched the photoluminescence of the QD. Once delivered into the cell, the QD-bound RNA probe hybridised with the pre-miRNA of interest. This complex was supposed to be cleaved by Dicer in a comparable manner to native conditions. As a result, QD and Au-NP were separated, leading to the detectable fluorescence emission of the QD.\textsuperscript{303}
One drawback of this design is the fact that binding of a complementary strand to the pre-miRNA of interest most likely changes the native pre-miRNA structure drastically. Dicer might rather recognise the antisense-pre-miRNA hybrid double strand than the pre-miRNA itself, as it would be intended. Furthermore, for mRNA it has been shown that the RNA is packed in granules for targeted transport within a neuron.\textsuperscript{106,304,305} If this is also true for pre-miRNA, then packaging might be impaired due to the binding to bulky and highly modified complementary strands. Proteins such as a DEAH box helicase might not be able to bind to the loop region and decipher the intended localisation, as it has been shown for pre-miRNA-134.\textsuperscript{233} For these reasons, two different designs of a pre-miRNA-181 derivative have been developed, that do not have the same drawbacks as the previously discussed probes. These probes were synthesised and tested with a variety of different dye combinations.

In both designs, the two dye modifications are separated from each other upon Dicer processing. Table 5 shows the different fluorophore-quencher pairs, their molecular structure as well as excitation and emission wavelengths of the respective fluorophores. Except for the Pyrene-Perylene probe, all used quenchers are dark quenchers and show no fluorescence themselves. The probes are named according to the fluorophore quencher pair used.
**Table 5:** Overview of pre-miRNA-181a1 probes that have been designed for this study. Different fluorophore-quencher pairs have been tested. Their chemical structures are shown as well – the fluorophore is shown above and the respective quencher underneath.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>λ&lt;sub&gt;ex&lt;/sub&gt; / λ&lt;sub&gt;em&lt;/sub&gt; in nm</th>
<th>Quencher</th>
<th>Dye &amp; quencher structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fluorescein-Dabcyl probe</strong></td>
<td><strong>Fluorescein</strong></td>
<td>485 / 514 (pH 9)</td>
<td>Dabcyl</td>
</tr>
<tr>
<td><strong>TAMRA-BHQ2 probe</strong></td>
<td>TAMRA</td>
<td>546 / 576</td>
<td>BHQ2</td>
</tr>
<tr>
<td><strong>Pyrene-Perylene probe</strong></td>
<td>Pyrene</td>
<td>300-350 / 350-400</td>
<td>Perylene</td>
</tr>
<tr>
<td><strong>tC₀ -tC&lt;sub&gt;nitro&lt;/sub&gt; probe</strong></td>
<td>tC₀</td>
<td>360 / 465</td>
<td>tC&lt;sub&gt;nitro&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

Fluorescein was used, even though its photochemical instability is known, but it can be easily incorporated during SPS and combined with the dark quencher Dabcyl. After optimisation of *in vivo* studies it could be replaced by more stable dyes such as Atto488 or AlexaFluor488, which are strongly related to Fluorescein with the same excitation and emission wavelength range, even though these dyes would need post-synthetic labelling of the probe. Like Fluorescein, Dabcyl can also be introduced into an oligonucleotide as phosphoramidite during SPS and is the least sterically demanding dark quencher. In contrast, the more stable fluorophore TAMRA (abbrev. tetramethyl-6-carboxy rhodamine), which is closely related to Rhodamine2b, should be used with the more bulky BHQ2 quencher.

Pyrene and Perylene are well-known and rather small fluorophores. Apart from the rigid alkyne-based covalent connection to the nucleobase, the photochemical properties of
Pyrene might be a disadvantage. It is excited at rather blue-shifted wavelength between 300 and 350 nm. Apart from the sensitivity of biological systems against light of higher energies, this dye could not be combined with common NB-based PPGs, if this would be intended in future steps of the project. Furthermore, the quantum yield $\phi$ of Pyrene averages 0.3. For comparison, the quantum yield of Fluorescein is 0.8.

As a last modification, the so-called tC$^O$-tC$^{nitro}$ fluorophore-dark quencher pair – both tricyclic cytosine analogues – was introduced into the sequence of pre-miRNA-181a1. While the photochemical properties of tC$^O$ are clearly inferior ($\lambda_{ex} = 360$ nm, $\phi = 0.2$), the advantage of this dye and its quencher is the perfect mimic of a natural cytosine base, so structural impairments are not expected.

It should be noted that all modifications were used as DNA modifications within the pre-miRNA-181a1 sequence. It needed to be tested whether two DNA modification close to the cleavage site were accepted by Dicer. If not, the probe architecture would need rearrangement or modified RNA phosphoramidites would need to be synthesised.

The sequence of the native pre-miRNA-181a1 is shown below. Interestingly, very similar mature miRNA-181 can result from a number of precursor isoforms (a1, a2, b1, b2, c, d). For this study, the pre-miRNA-181a1 isoform of rat (*rattus norvegicus*, Rno) is exclusively used.

![Figure 116: Sequence and secondary structure of native pre-miRNA-181a1. The 5’-end contains a terminal phosphate indicated as P. After Dicer processing the mature miRNA-181a exists initially as double strand with the miRNA-181a* passenger strand.](image)

All probes for this study were synthesised using SPS with 2’-tBDMS RNA chemistry. In this case, 2’-modifications such as 2’-O-methyl- or 2’-fluoro-RNA as RNase protecting were not indicated, since RNase digestion, in particular Dicer-mediated processing, is clearly intended.

In a first synthesis approach, enzymatic ligation of two shorter RNA sequences seemed useful, as synthesis of the whole sequence of 63 nucleotides was not thought to result in sufficient yields. To optimise ligation, the synthesis was first executed without any fluorescent modifications. Therefore, the whole sequence was divided into two parts within the loop region. To make ligation possible, one strand needs to contain a 5’-end phosphate moiety. The phosphate group can either be attached enzymatically or during solid phase synthesis. Enzymatic phosphorylation is known to result in lower yields than using the commercially available chemical phosphorylation agents for SPS.
The phosphate is generated by the β-elimination reaction of a diethylsulfonyl group, as shown in Figure 117. It is an inexpensive and versatile reagent that can be used for 5'-phosphorylation, as well as generating a 3'-phosphate, if used as solid support.

It is also recommended to use a so-called splint DNA to increase ligation yields. The splint DNA is complementary to the two RNA strands in the region of ligation. Like that, the two endings are kept in close proximity to each other.

One massive drawback of enzymatic ligation is the possibility of side reactions such as entropically favoured ring closures. Native pre-miRNAs naturally contain phosphate groups at their 5'-end due to previous Drosha-mediated processing. This phosphate could wrongly be used for ligation. For this reason, this naturally occurring phosphate was planned to be attached enzymatically after ligation.

For ligation, all three strands are incubated at 90 °C for 3 min and cooled down slowly to allow for formation of the most stable structure. Afterwards, T4 ligase was added and the mixture was incubated at 33 °C for 120 min. The reaction mixture was buffered according to manufacturer’s protocol. Subsequently, the oligonucleotides were isolated using standard chloroform extraction and purified via anion exchange HPLC. This procedure was repeated several times, but never yielded in sufficient amounts of the full-length pre-miRNA-181a1.

As a consequence, it was decided to synthesise the modified probes on the whole via SPS. Against most expectations, the synthesis of the different RNA-63mers worked well with yields up to 55%. SPS and deprotection could be executed without any changes compared to the synthesis of shorter sequences. HPLC runs had to be adapted, since longer sequences elute massively later in AE-HPLC and, due to secondary structures, only slightly later in final RP-HPLC purification.

After purification, the probes were heated up to 95 °C for 5 min followed by rapid cool-down on ice to ensure the formation of the most stable secondary structure.
In a first *in vitro* test, all designs were incubated with different types of recombinant human Dicer enzymes. Unfortunately, the results were not satisfying for neither of the tested commercially available Dicer. The results will be discussed below.

**Table 6: Commercially available recombinant Dicer kits, the according manufacturer and the recommended incubation time.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Manufacturer</th>
<th>Incubation time at 37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>TurboDicer™ siRNA Generation Kit</td>
<td>Genlantis</td>
<td>max. 2 h</td>
</tr>
<tr>
<td>Recombinant Dicer Enzyme Kit</td>
<td>Genlantis</td>
<td>12-18 h</td>
</tr>
<tr>
<td>PowerCut Dicer</td>
<td>Finzymes</td>
<td>18-24 h</td>
</tr>
</tbody>
</table>

The probes were incubated with the enzyme according to manufacturer’s protocol. TurboDicer by Genlantis is a recombinant human Dicer that is supposed to be ultra-active and to cleave 95% of all double-stranded RNAs into 22-nt long oligonucleotides. According to manufacturer, unsuccessful reaction can mostly be explained by an excessive amount of enzyme or hydrolysed ATP solution. Both reasons can be excluded in this case, as the tested amounts of enzymes as well as incubation times were varied without any change in reaction efficiency (data not shown).

In contrast to TurboDicer with a reaction time of 2 hours, the common Dicer enzyme kit by Genlantis recommends a reaction time between 12-18 h. The manufacturer states that the efficiency of this enzyme kit is fully independent from the species the RNA originates from. Nevertheless, it is recommended to use templates between 500 to 1000 bp, since the enzyme is known not to digest well, if dsRNAs below 300 bp are used. In contrast, the studied pre-miRNA probes have a total length of approx. 60 bp in a stem-loop structure.

The third tested enzyme was PowerCut Dicer by Finzymes. The enzyme derives from *Giardia intestinalis* and is supposed to be 100% efficient for dsRNA between 200 and 800 bp. However, a study by MacRae et al. in 2012 shows that even this Dicer variant becomes less active below 500 bp. Nevertheless, this enzyme has the advantage of being very robust and producing less side products.  

In summary, it can be concluded that most commercially available Dicer enzymes are not suitable for processing the here presented pre-miRNA probe due to its short sequence. While human Dicer enzymes are not efficient at all at this length, bacterial Dicer might be an alternative. Bacterial Dicer has the massive disadvantage of being highly unspecific. Fragments between 30 and less than 10 nucleotides can be found after digestion with bacterial Dicer. In this reason, it was chosen to continue the study with neuronal cell lysate, as previous studies had already proven that cell lysates are usually more efficient and specific than purified Dicer enzymes. In none of the reactions with above mentioned enzymes, a fluorescence increase above 2x compared to the background was observed (cf. Figure 119). Nevertheless the results of the different pre-miRNA probe digestions confirmed that the probe might work in general. Even though fluorescence increase was not significant, the
Local miRNA Maturation

results seem to suggest that pre-miRNA probes with fluorophore and quencher on different sides (A-probes) might be digested more efficiently. For clarity reasons, exclusively results of the Fluorescein-Dabcyl probe are shown. The results of other probe designs, especially the TAMRA-BHQ2 probe, do not differ from the results for Fluorescein-Dabcyl probes shown below.

Figure 119: Results of Fluorescein-Dabcyl pre-miRNA181a1 probes with different commercially available recombinant human Dicer enzymes. Design A (lighter colour) has Fluorescein and Dabcyl on opposite sides of the stem-loop structure, while design B (respective darker colour) has the two modifications on the same side. The maximal fluorescence increase is approx. twice as high as the background fluorescence (indicated by a black line, set to 1). It seems, as if exclusively design A can be processed by Dicer.

Lack of fluorescence increase could be explained by incorrect cleavage as well, if fluorophore and quencher were not separated. This possibility was excluded by subsequent chloroform extraction and RP-HPLC analysis of the Dicer reaction mixtures. The RP-HPLC analysis confirmed that the probes were either not or hardly digested, as fluorescence measurements had previously suggested.

Furthermore it was hypothesised that Dicer might not be able to digest 60nt-short stem-loop structures without TRBP (abbrev.: trans-activating response RNA-binding protein). This protein has been shown to be an essential part of the Dicer complex. It contains three binding sites for double stranded RNA. It is also known that TRBP is required for later Ago2 recruitment for RISC formation. As a consequence, TRBP knock-down leads to a loss of miRNA biogenesis due to Dicer complex destabilisation. For this reason, it was continued to use cell lysate of hippocampal rat neurons to make sure that all necessary “chaperonic” proteins were present for optimal Dicer action. Furthermore,
the possibility that human recombinant Dicer might not be able to process the rat sequence of pre-miRNA-181 derivatives was likewise excluded.

Cultured rat neurons (1 Mio., 20-30 days in vitro (DIV)) were lysed in either PBS (1x) or Dicer buffer using cell scraper and syringes (cf. methods). Approximately 10,000 cells were then used for 5 nmol of each probe. The fluorescence increase was measured several times within 48 h and was based on the fluorescence of undigested control probes with their fluorescence set to 1. As a result, it could be concluded that exclusively probes with their fluorophore and quencher on opposite sides could be processed, as it had been observed previously for recombinant Dicer. Besides, the Pyrene-Perylene and tC^OtC^nitro probes both resulted in comparably low fluorescence increases of only 2.5 and 3 compared to the undigested control probe (data not shown). Because their SNR was rather low and regarding the fact that their excitation wavelength is blue-shifted compared to most dyes, these probes were not further investigated. For both Fluorescein-Dabcyl and TAMRA-BHQ2 probes, the signal increase was found to be up to 9 compared to the background fluorescence of undigested probe. Despite their bulky modifications and the presence of DNA modifications, the probes were well accepted by Dicer, as long as their modifications were attached on opposite sides of the pre-miRNA probe (Design A, cf. Figure 120). One could conclude that the modifications might interfere with Dicer binding sites, when they were attached on the same side.

Even though the results for TAMRA and Fluorescein looked promising, it was still possible that fluorescence increase was caused by unspecific RNase-mediated digestion. To exclude this possibility, the experiment was repeated with varying amounts of RNase inhibitor (RI, RNasin®, Promega). Worth mentioning, RNasin does not inhibit Dicer activity, since – unlike most RNases – Dicer belongs to the RNase type III family and therefore is not targeted by standard RIs.
Figure 121: Fluorescein-Dabcyl and TAMRA-BHQ2 pre-miRNA-181a1 probes (only design A) were incubated with neuronal cell lysate either with or without (w/o) RNase inhibitor (RI, 1:100 dilution). No difference can be observed. It can be concluded that no unspecific RNase-mediated digestion occurs, when the probes are incubated with neuronal cell lysate. After 24 h, maximal fluorescence seems to be reached.

The results depict that there is hardly any difference between the reaction with or without RI. It was concluded that RNase-mediated, unspecific digestion of the pre-miRNA probes could be neglected during incubation with neuronal cell lysate.

Additional proof of Dicer-mediated cleavage would be the specific inhibition of Dicer and, consequently, decreased fluorescence signal of the probe. In one of his studies, ARENZ and his group identified three peptides mimicking short sections of the Dicer amino acid (AA) sequence of 12 AA length. The chosen sequence sections either derived from the RNase or helicase domain of Dicer. The most promising of these peptides named S186 was chosen and aligned with the Dicer AA sequence of rat. S186 is part of the helicase structure in Dicer and was found in the sequence of rat Dicer as well. The according sequence is N-AKPYSQRRKTSQ-C. Notably, in ARENZ' studies, the N-terminus was acetylated and the C-terminus was chosen as amide instead of the free carboxyl acid.

Preliminary in vitro tests with neuronal cell lysate according to the published protocol by ARENZ resulted in signal decrease down to 0.8 compared to uninhibited positive control without peptide S186. Increase in peptide concentration could further decrease fluorescence down to a minimal value of 0.6 compared to the positive control without inhibition. Nevertheless, full inhibition of Dicer or even comparable values to the published data by ARENZ et al. (decrease to 0.2 compared to the uninhibited control) could not be observed. It was already expected, that the change in fluorescence might not be distinct enough for significant cellular results.
Figure 122: Effect of peptide S186 on Dicer activity. This peptide sequence has been shown to inhibit Dicer processing of pre-miRNAs by Arenz et al. The negative control (red) does not contain neuronal cell lysate in contrast to all other probes. After 48 h, a probe with 100 µM peptide concentration (green) shows approx. 80% of the maximal fluorescence, if no peptide is added (yellow). 200 µM (blue) and 500 µM (purple) peptide decrease fluorescence increase to approx. 60%.

Another approach to inhibit Dicer indirectly is the usage of secondary structure binders. These organic small molecules bind specifically to certain secondary structures of RNAs such as bulges or loop sequences. To date, a number of these secondary structure binders are known, most of them are commonly used as antibiotics, as the binding of the molecule to the RNA can inhibit further enzymatic processing. After choosing the most promising among them that are not fluorescent themselves in the range of TAMRA or Fluorescein, in vitro tests with neuronal cell lysate were performed. After 24 h of incubation at 37 °C, it could be observed that all secondary structure binders had an effect on Dicer cleavage efficiency. Two of the reactions even showed negative fluorescence results. This finding might be due to interaction of the small molecule with the fluorophore and a quenching effect. For data analysis, a non-inhibited control was set to 100%.

Figure 123: Screen for small molecules that are known to bind to secondary structures of RNA and consequently might be able to inhibit Dicer processing of pre-miRNA-181a. The experiment was performed with neuronal cell lysate and fluorescence was measured after 24 h. All secondary structure binders were used in 1 mM concentration. The best effect was observed for Neomycin. Negative fluorescence results might be due to quenching effects of TAMRA below the background fluorescence.
The most promising representative of all tested molecules was Neomycin, an aminoglycoside antibiotic that consists of several amino sugars connected via glycosidic bonds. Neomycin has a very potent effect against gram-negative bacteria. To date, its medical use is discussed controversially due to its nephrotoxic effects, especially when applied intravenously. Nevertheless, it is still in use in molecular biology for the selection and establishment of stable cell lines that have been made Neomycin resistant. Amino glycosides in general are known for their ability to bind to duplex RNA. Neomycin, as one of the most potent ones, has been shown to bind tightly to rRNA at the A-site within ribosomes (site of amino-acid-loaded tRNA binding) with a binding constant in the range of $10^9$ M$^{-1}$. Notably, no effect on DNA duplexes have been discovered.$^{312,313}$

In a further experiment, the necessary concentration of Neomycin was optimised. The optimal concentration seemed to be 1 mM of Neomycin within the reaction mixture. Further concentration increase was not successful in decreasing the fluorescence signal anymore. In this amount, Neomycin was able to suppress the fluorescence signal down to 2% compared to the uninhibited control, which was set to 100% fluorescence.

To prove that the decrease in fluorescence was not caused by quenching effects of Neomycin, the reaction was repeated with a TAMRA control probe. This control probe is modified with the TAMRA-dT as the regular TAMRA-BHQ2 pre-miRNA, but does not contain a quencher. As a result, the fluorescence should not differ between processed and intact (unprocessed) state. This positive control will also become important for later in vivo tests in living neurons to optimise concentrations. The results clearly show that no change of TAMRA fluorescence intensity was observed, when Neomycin was added at its optimal concentration. The fluorescence of the TAMRA control probe without Neomycin addition was set to 100%.

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**Figure 124**: a) Screen for the optimal Neomycin concentration to inhibit Dicer-cleavage of the TAMRA-BHQ2 pre-miRNA-181a probe as efficiently as possible. The experiment was performed with neuronal cell lysate and fluorescence was measured after 24 h. A concentration of 1 mM blocks fluorescence increase almost completely. To exclude the possibility that Neomycin does not inhibit Dicer cleavage, but rather quenches TAMRA fluorescence, the experiment was repeated with a control probe that does not contain the dark quencher BHQ2 (structure shown in c)) b) Molecular structure of Neomycin, R can be H or CH$_2$NH$_2$. 

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The final and most convincing proof of successful Dicer-mediated cleavage of the optimal probes was thought to be an experiment with Dicer knock-out (KO) cells. It should be noted, that Dicer-lacking animals such as mice have shown to die early during either embryonic or post-natal development. In contrast to other species, mammals most likely have a single Dicer gene and its knock-down has been shown to cause severe defects in miRNA and short hairpin RNA (shRNA) generation and consequently prevent the RNAi mechanism. In most organisms, global inactivation of Dicer often results in growth arrest and developmental block. Especially in mice, Dicer depletion leads to embryonic lethality. Lethality is mostly explained by cardiac defects due to the depletion of the muscle-specific miRNA-1 in mice during embryonic development. But also other cell defects of lymphocytes and defects in blood vessel development in embryonic mice have been proven. In 2013, Krill et al. managed to generate mice that were lacking Dicer exclusively within the adrenal cortex. The adrenal cortex is part of the adrenal gland and therefore important for hormone production such as cortisol and aldosterone. Among others, they regulate stress response and electrolyte homeostasis. These mice showed first defects and also mortality between the embryonic stage E16.5 and E18.5.

Dicer-deficient embryonic stem cells can be generated by gene-targeting approaches, as it has been described by Kanellopoulou et al. For this study, we gratefully received cells of Dicer KO (Dicer1<sup>shhICre</sup>) mice from the group of Prof. Rohrer from the Max Planck Institute for Brain Research in Frankfurt. These cells derived from neuronal ganglia of either the embryonic E16.5 or the post-natal P1 state. Empirically, approx. 50% of the cells were expected to be Dicer-lacking. Quantitative PCR analysis determined which cellular lysate derived from Dicer KO mice. As before, the cell lysate was incubated with the TAMRA-BHQ2 probe in presence of high RNase inhibitor concentrations (1:25 (v/v) dilution). After 24 h at 37 °C incubation time, the fluorescence increase was determined.

For data evaluation of the two experiments with embryonic or post-natal state cells, the maximal fluorescence of the unquenched TAMRA control probe (see structure above) was set to 1. The respective florescence increase caused by Dicer KO cells or Dicer-containing cells (Dicer wild-type), respectively, were set in relation. The specificity of the signal was analysed with the t-test to calculate the specificity of the results. In both cases – for embryonic and post-natal cells – the difference was determined as extremely and very statistically significant, respectively. The fluorescence signal was five times lower in Dicer KO cells compared to Dicer wild-type cells.
After optimising and verifying the function of the probe design, the choice of the fluorescent dye was supposed to be optimised. Most superior dyes are not available as phosphoramidite for direct use in SPS. The commercially available fluorescent phosphoramidites such as xanthene and cyanine derivatives are usually not stable enough for tracking experiments or might not be suitable for the microscopy technique of choice. Additionally, if red-shifted fluorophores are needed due to e.g. auto-fluorescence of cells or because red-shifted light is less harmful and able to penetrate tissue much deeper, these fluorophores are not available as phosphoramidites due to their instability against basic conditions. Even charges of the dye might be important for the application. Positively charged probes have been shown to efficiently target the nucleus of a cell, while probes with a negative charge tend to stay in the cytoplasm, independently from size.321 To make the pre-miRNA probes suitable for a wide range of applications, several post-synthetic labelling strategies were tested. For this reason, the according functional groups were introduced co-synthetically into the probe at the position, where the dye was supposed to be and that has been shown to be a suitable position in the previous studies discussed above. First, a common amino modified dT analogue was introduced. BHQ1-dT was used at the respective position on the opposite side, as before.

After optimising and verifying the function of the probe design, the choice of the fluorescent dye was supposed to be optimised. Most superior dyes are not available as phosphoramidite for direct use in SPS. The commercially available fluorescent phosphoramidites such as xanthene and cyanine derivatives are usually not stable enough for tracking experiments or might not be suitable for the microscopy technique of choice. Additionally, if red-shifted fluorophores are needed due to e.g. auto-fluorescence of cells or because red-shifted light is less harmful and able to penetrate tissue much deeper, these fluorophores are not available as phosphoramidites due to their instability against basic conditions. Even charges of the dye might be important for the application. Positively charged probes have been shown to efficiently target the nucleus of a cell, while probes with a negative charge tend to stay in the cytoplasm, independently from size.321 To make the pre-miRNA probes suitable for a wide range of applications, several post-synthetic labelling strategies were tested. For this reason, the according functional groups were introduced co-synthetically into the probe at the position, where the dye was supposed to be and that has been shown to be a suitable position in the previous studies discussed above.

First, a common amino modified dT analogue was introduced. BHQ1-dT was used at the respective position on the opposite side, as before.
After normal work-up, the labelling strategy that had worked very well in the previous Molecular Beacon project for amino group labelling was applied. In this case, either AlexaFluor488 or Atto488 were used as NHS ester for labelling. After the reaction, HPLC analysis revealed that no labelled oligonucleotide was obtained at all. For further optimisation, a number of conditions were tested. Always one condition was changed per reaction.

1. The equivalents of dye relative to the oligonucleotide were varied: 1 eq., 1.5 eq., 2.5 eq.
2. Reaction time: 90 min, 120 min, overnight
3. pH: 8.0, 8.3, 8.5, 9.0
4. Temperature: RT, 30 °C, 40 °C

None of these reaction conditions resulted in improvement. No labelled probe was obtained at either condition. It was not even possible to successfully label sufficient amounts for analysis. Some of these conditions were already expected not to work, since hydrolysis is known to compete with the labelling reaction. High pH values and elevated temperatures would favour the hydrolysis reaction.

To overcome the issue of hydrolysis, maleimide-modified dyes were used for labelling.

![Alternative Labelling strategy of amino modified oligonucleotides with maleimide-functionalised dyes (Atto488). This reaction is commonly known to be a side reaction of the maleimide labelling strategy at basic pH values, if aliphatic amino groups are present.

This reaction is usually known as undesired side reaction, if both thiol and amino modifications are present within an oligonucleotide. The thiol maleimide labelling is commonly conducted at pH 7, because amino groups are still protonated at this pH until approx. pH 8. At elevated pH values, aliphatic amino groups are nucleophilic enough to react in an addition reaction in an equivalent way thiol groups do.

Unfortunately, this modified labelling reaction did not work either. No labelled compound was achieved at all.

Another approach of labelling amino modified oligonucleotides is the usage of more stable active esters such as SDP or TFP, that have been briefly discussed in the introduction chapter. Some AlexaFluor dyes are already available with these modifications to date. Especially the SDP ester is currently the most stable amino reactive moiety against hydrolysis. After labelling, the same strong amide bond between the dye and the oligonucleotide is produced. SDP- and TFP-reactions can even be performed at elevated temperatures and basic pH values up to 9.0. Nevertheless, neither Alexa488-TFP nor the respective SDP ester yielded in any fluorescently labelled pre-miRNA-181a1 probe, when applied according to manufacturer’s protocol.

Alternatively, a new oligonucleotide was synthesised with a thiol-modified dT at the respective position. Even the thiol-modified reaction with Atto488-maleimide did not yield any dye-labelled pre-miRNA-181a1 probe.
In literature, it has been claimed that long RNA oligonucleotides with complex secondary structures are rather difficult to label. However, the authors successfully labelled a 70mer internally by CuAAC click chemistry. In this study, the authors successfully screened a number of different reaction conditions regarding solvents, equivalents, and reaction times.\(^{57}\)

First, alkyne-labelled pre-miRNA-181a\(^1\) analogues had to be synthesised with the modification at the respective positions, where the fluorophore is supposed to be attached as before. Two different alkyne modifications have been tested.

The following reaction condition has been the most successful one in the study by Pradère et al.\(^{57}\) and has therefore been applied in this study for the pre-miRNA-181a\(^1\) labelling as well. All labelling reactions were conducted under inert gas in an oxygen-free atmosphere. This procedure is not mandatory, if a TBTA (tris-(benzyltriazolylmethyl)amine) ligand is used in the reaction, as it reliably stabilises the Cu(I) oxidation state.
Table 7: Reaction condition for CuAAC click chemistry labelling of alkyne-labelled pre-miRNA-181a1.

<table>
<thead>
<tr>
<th>Atto488-azide</th>
<th>Cu(II) source</th>
<th>Ligand</th>
<th>Organic solvent</th>
<th>Rct. time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 eq</td>
<td>CuSO$_4$ 5H$_2$O</td>
<td>TBTA (25 mM final conc.)</td>
<td>MeOH:DMF (1:1, v/v)</td>
<td>16 h (o/n)</td>
<td>45 °C</td>
</tr>
</tbody>
</table>

For C8-Alkyne-dU, the yield of Atto488-labelled pre-miRNA probe was approx. 43%. The probe performed similarly to Fluorescein and TAMRA probes in _in vitro_ tests. The labelling yield of the 2'-O-propargyl uridine modified pre-miRNA derivative was much lower with approx. 5%. Even the SPS yield had already been inferior. For this reason, the 2'-O-propargyl uridine-modified probe was not further analysed.

For C8-Alkyne-dU-BHQ1-modified probe after Atto488-azide-labelling and upon incubation with neuronal cell lysate. The fluorescence increase is set relatively to an undigested positive control (background fluorescence set to 1). The probe was labelled post-synthetically with a CuAAC click chemistry reaction. Afterwards, the successfully labelled probe was incubated with neuronal cell lysate (DIV 28) in the presence of RNase inhibitor, as it has been explained above.

Preliminary _in vivo_ tests showed, though, that the usage of Atto488 was not necessary. Especially TAMRA-labelled pre-miRNA-181a1 probes performed very well, as it will be discussed in the next subchapter. Additionally, the synthesis of TAMRA-labelled probes was much faster with a higher overall yield, as the post-synthetic labelling step was not necessary. For this reason, all final _in vivo_ experiments were exclusively performed with co-synthetically labelled probes.
Visualisation of pre-miRNA Processing in vivo

The following in vivo experiments were conducted by the group of Prof. Erin Schuman from the MPI for Brain Research, Frankfurt.

Once the principal function of the probe had been proven in vitro, in vivo tests with hippocampal rat neurons were planned. Initial experiments were conducted using the Fluorescein-Dabcyl probe. The TAMRA-BHQ2 pre-miRNA181a1 probe was later used to overcome the issue of autofluorescence of the neuron.

One prerequisite for dendritically localised miRNA maturation upon signal alteration is the presence of Dicer in neuronal dendrites. Figure 131 shows the result of an immunostaining experiment to confirm dendritically distribution of Dicer. The first antibody was Dicer-specific and the second fluorescent one targeted the primary antibody. By comparison with the MAP2 (Microtubule-associated protein 2) staining, it is obvious that Dicer is evenly distributed in dendrites, independently to the distance from the soma.

A negative control confirmed that this binding is specific, because in the absence of the Dicer-specific primary antibody, the background fluorescence was very low and unspecifically distributed.

MAP2 staining is a common method to visualise neuronal dendrites. MAP2 is known to stabilise microtubules and – among others – is responsible for the dendritic shape during developmental steps.322

Figure 131: In green, the MAP2 staining is shown for the respective neuron. Like this, neuronal dendrites can be visualised. In blue on the left side, the immunostaining against Dicer is shown. It is obvious that Dicer is evenly distributed within the soma and along the dendrites. On the right side, the background fluorescence is shown, when the primary, Dicer-specific antibody is not used.

It was also important to know whether native pre-miRNA-181a1 is present in dendrites. Only if this is the case and pre-miRNA-181a1 is actively transported to the dendrites, local, Dicer-mediated maturation can be expected. In literature, several pre-miRNAs have been identified that are located in the soma, among them pre-miRNA-150 and potentially pre-miRNA-124.163,228
To demonstrate dendritic distribution of pre-miRNA-181a1, a high resolution FISH experiment was conducted. For this purpose, a fluorescently labelled strand that was complementary to the loop region of pre-miRNA-181a1 was chosen. Figure 132 depicts the results of the FISH experiment. Obviously, the highest signal can be found in the soma including the nucleus. It should be noted that especially the nuclear signal may also result from the respective pri-miRNA, as pri- and pre-miRNAs share the same loop region. More importantly, the FISH signal also co-localises with the dendritic MAP2 signal. This proves the presence of native pre-miRNA-181a1 in dendrites in a punctate structure. It could even be discussed, whether the pre-miRNA-181a1 signal might be slightly adjacent to the MAP2 signal and is mostly located in dendritic spines. This fluorescent signal is not expected to arise from pri-miRNA, as these structures are solely exported from the nucleus after Drosha processing regarding current knowledge.

In the case of a scrambled FISH probe, only background fluorescence is visible and the signal does not co-localise with the dendrites. Same is true for a negative control with no fluorescent FISH probe present.

Figure 132: Results of the high resolution FISH experiment to prove the presence of native pre-miRNA-181a1 in dendrites. The FISH probe was complementary to the loop region and seven additional bases of the pre-miRNA-181a1. The target sequence section is shown in purple (indicated in a). Notably, the pri-miRNA-181 shares the same loop region and therefore a high fluorescent nuclear signal is not unexpected. In addition to somatic localisation, the pre-miRNA-181a1 co-localises with the MAP2 signal of the dendrites (left). In contrast, if a scrambled FISH probe (middle) or no fluorescent probe (right) at all are used, there is only background fluorescence signal that is not co-localised with dendrites.

In the next step, the dually labelled pre-miRNA-181a1 probe had to be delivered to the neurons to visualise its processing in vivo. On the basis of GERHARD SCHRATT's study, two transfection techniques were tested, namely Magnetofectamine (OZ Biosciences) or LipofectAmine 2000 (Invitrogen). Neither transfection method resulted in acceptable microscopy images. The main issue was the presence of fluorescent precipitates that outshone the dendritic signal. To remove the fluorescent precipitates, a number of possible solutions were tried. First, the washing procedures during transfection were prolonged or performed several times more than the
transfection protocol recommended. Furthermore, a so-called cell-scrub buffer (Genlantis) was used. The recipe of this buffer has not been disclosed by the company, but the buffer is supposed to remove extracellular DNA complexes associated with cell or plate surfaces during transfection. Therefore, a more reliable analysis of the transfection result was expected. Unfortunately, the usage of cell-scrub buffer did not show any improvement. It was hypothesised that the precipitation may form due to the positively charged plate coating the neurons grew on. Changes in the coating resulted in dying neurons. Furthermore, the auto-fluorescence of the neurons corresponded to the fluorescence emission of Fluorescein. Even untransfected cells used as negative control seemed to show the same fluorescence as the transfected ones.

![Figure 133: Results of transfection (Magnetofectamine) of the Fluorescein-Dabcyl pre-miRNA-181a1 probe. The MAP2 stainings of the respective neurons are shown in red. Fluorescent precipitates outshine the dendritic signal. Furthermore, the auto-fluorescence of the cells seems to show a similar dendritic signal, even though no probe is added. Neither improvement of the washing steps during transfection nor changes in the transfection protocol could remove the precipitates.](image)

To see whether there is an observable difference when Dicer is blocked, poly-L-lysine (PLL), which has been sown in literature to inhibit Dicer, was used. As the peptide S186 had not shown a convincing inhibition in in vitro assays, PLL was tested in vivo according to published procedures. Importantly, an accurate determination of the PLL concentration is generally rather difficult due to the broad molecular weight range of 4,000-15,000 g/mL of the polymer.

In the published study by WATASHI et al. no toxic effect of PLL was observed. Nevertheless, in this study, all neurons died under PLL treatment and fluorescence was rather increased than decreased, most likely due to RNase activity during cell apoptosis. Under comparable detection settings for Fluorescein and MAP2, no MAP2 signal was observed, indicating a dying, unhealthy cell. The gain had to be increased extremely to see the MAP2 signal within the background.
Figure 134: Fluorescence signal of the Fluorescein-Dabcyl probe (green) after transfection in the presence of PLL that has been shown to inhibit Dicer activity. If the gain of the microscope is set to comparable values for probe and MAP2 staining, no MAP2 signal is visible indicating a dying neuron. Only massive increase of the gain makes the MAP2 staining visible within the background.

Even though most transfection experiments were not significant due to fluorescent precipitates, it was possible to achieve some preliminary data with the TAMRA-BHQ2 probe. The advantage of TAMRA is the fact that its fluorescence emission does not correspond to the neuronal auto-fluorescence. The results are shown in Figure 135.

To estimate the maximally fluorescence value, a positive control without quencher was transfected (Figure 135 A). The dual-labelled pre-miRNA-181a1 probe that fluoresces exclusively after Dicer processing, shows a punctate structure within the dendrite that might reflect the locally restricted processing (Figure 135 B). Due to the precipitation issue, this result must be seen as preliminary and signal increase due to precipitates in this area cannot fully be excluded.

As mentioned above, the pre-miRNA-150 has been shown to be exclusively somatically located. Furthermore, the localisation has been shown to be mediated by the sequence of the loop region. On the basis of this study by Schratt et al., a hybrid pre-miRNA was designed. This hybrid combined the loop region of pre-miRNA-150 with the stem structure of pre-miRNA-181a1. Interestingly, even the preliminary data supported the findings by Schratt et al. A decreased dendritic signal was found in the dendrites in the case of the hybrid pre-miRNA (Figure 135 C).
Local miRNA Maturation

Figure 135: Dendritic signal of A) Positive control of quencher-free probe reflecting maximal fluorescence. B) TAMRA-BHQ2 probe indicating a punctate fluorescent structure that might be caused by locally restricted Dicer activity. C) Hybrid pre-miRNA with the loop sequence of the somatically localised pre-miRNA-150 combined with the stem structure of pre-miRNA-181a1. This hybrid pre-miRNA is hardly found in dendrites.

In addition to the technical issues of transfection regarding massive precipitation, the time frame of transfection seemed to be too long in comparison to Dicer activity. Most transfection techniques take at least 30 min up to several hours, before cells can be analysed. For this reason, the patch clamp technique was chosen as alternative approach that allows for real-time visualisation of Dicer-dependent probe processing.

For visualisation of neuron morphology, AlexaFluor488 azide was co-introduced into the cell. To determine optimal probe concentration for the visualisation of distant dendrites, a quencher-less positive control was used as before. The optimal concentration was found to be 20 µM.

Initial experiments showed that – with the chosen concentration – probe processing became visible after approx. 2 min. After 15-30 min, fluorescence was well visible in soma and dendrites. Even though fluorescence became also visible in dendrites, the highest fluorescence signal was found in the soma. Regarding the results, it was not possible to distinguish between fluorescent mature miRNA-181, which had been processed in the soma and diffused along the dendrites, and dendritically Dicer-mediated processing.

In another experiment, neurons were stimulated in order to visualise how Dicer activity changes. For this reason, Cs⁺ gluconate (120 mM) was added to the intracellular (patch) solution. Cs⁺ ions can block K⁺ currents and consequently increase depolarisation. As a result, fluorescence increase was massively accelerated. Comparable fluorescence values of unstimulated neurons after 30 min have already been found after 10 min due to stimulation. After 25 min maximal fluorescence was reached in the soma. Again, it is not possible to exclude diffusion of somatically processed probes from the soma to the dendrites.

In a third experiment, (2R)-amino-5-phosphonopentanoate (APV) was added in addition to Cs⁺-mediated stimulation. APV blocks NMDA receptors by competing for the glutamate binding site.

Figure 136: Chemical structure of APV, a selective NMDAR inhibitor. APV competes for the glutamate binding site.
Interestingly, probe processing was neither detected in the dendrites nor in the soma. Exclusively the nucleus seemed to show fluorescent signal with the maximal value after approx. 25 min. As a consequence, it can be concluded that Dicer-mediated miRNA-181a1 maturation is not happening during depolarisation of the cell, when NMDARs are blocked. Pre-miRNA-181a1 processing seems NMDAR and Ca\(^{2+}\)-dependent, respectively.

Figure 137: The figures show the neuronal morphology, stained by an AlexaFluor488 azide (left) and the time-dependent processing of the inducible TAMRA-BHQ2 probe (right). In the first row, the basal activity is shown. Fluorescence slightly increases, mostly in the soma, but also in the dendrites. Upon stimulation, the Dicer-dependent processing seems massively accelerated. Again, the strongest signal can be found in the soma. The last row shows the effect of simultaneous stimulation and NMDAR blockage by APV. Interestingly, fluorescence increase in soma and dendrites is almost negligible. One exception is the nucleus.

Figure 138 makes it even more obvious. Upon stimulation, the Dicer activity increases in both dendrites and soma. The fluorescence increase in the soma is higher compared to dendrites. The somatic fluorescence is five times higher compared to basal levels after 30 min under Cs\(^{+}\)-supported stimulation. In contrast, the fluorescence is hardly twice as high as the basal value in the dendrites upon stimulation. Notably, the somatic and dendritic values arise from the same set of neurons under the respective experimental conditions.

One interesting fact should not be neglected. During APV-mediated NMDAR-blockage, the fluorescence in the soma is well comparable to the basal level. In contrast, regarding the dendrites, the fluorescence is even lower than in a fully untreated cell. In the latter case, the fluorescence did not seem to change at all and stays constant. This is another hint that the fluorescence increase in dendrites is strongly NMDAR-dependent. Furthermore, the difference between the APV-caused signal in soma and dendrites might indicate local Dicer-activity and neither diffusion nor unspecific probe digestion.
Figure 138: Graphic view of fluorescence increase upon TAMRA-BHQ2 probe processing. Upon stimulation, the Dicer activity increases in dendrites and soma. Notably, the fluorescence increase in the soma is higher compared to dendrites. In the case of APV-mediated NMDAR-blockage, the fluorescence decreases again. Interestingly, the somatic fluorescence is well comparable to the basal level. In contrast, the dendritic fluorescence is even lower than in a fully untreated cell.

To fully exclude the possibility of diffusion-caused fluorescence increase in dendrites, neurons needed to be locally stimulated at certain dendritic positions. This can be achieved by local uncaging of caged glutamate. To do so, glutamate is masked as 1-acyl-7-nitroindoline. This type of caged neurotransmitters has been used successfully in the investigation of synaptic processes for several years.

The following images show the experimental results. Uncaging locations are indicated by red squares in close proximity to the dendrite of choice. Again, TAMRA-BHQ2 probe is co-delivered to Alexa488 azide to stain the cellular morphology of the neuron. Upon glutamate uncaging, the resulting EPSCs (uEPSCs) are found to be well comparable to spontaneous, native EPSCs (sEPSCs).

Figure 139: The morphology of the neuron is visualised by Alexa488 azide (green, a)). Positions of laser pulses are marked by red squares (uncaging area minimum). The resulting EPSC (uEPSC, b)) after uncaging laser pulses are similar to spontaneous EPSCs (sEPSC, c)). Caged glutamate (molecular structure shown in d)) is purchased from TOCRIS.

Usually the chosen position underlie an uncaging train of 30 pulses of each 1 ms duration with a frequency of 1 Hz. Afterwards, the position was observed in real-time for several minutes. The following figure depicts the images acquired up to 175 s before uncaging was started. Obviously, the dendritic fluorescence is stable prior to uncaging, as it has already
been shown before. Once the glutamate uncaging train had started (t = 0 s), the fluorescence signal increases rapidly in a restricted area close to the uncaging position. Interestingly, approx. half a minute after uncaging, an adjacent spine seems to increase in size. Furthermore, fluorescence signal arises and stays constant for at least 10 min.

Figure 140: Real-time visualisation of the effect upon glutamate uncaging in dendrites. The minimal uncaging area is marked by a red square. The uncaging starts at t = 0 s and first spine growth is visible after 16 s. Fluorescence increase is obvious after 24 s. Interestingly, after 2.5 min fluorescence decreases again and the adjacent spine starts growing and fluorescing.

This finding is also reflected by the following graph. Upon uncaging, the fluorescence increases fast, but finally drops to a constant level. At this time point, the fluorescence increase of the neighbouring spine can be observed. Several publications have already addressed this issue of how restricted local processes in neurons might be. Most of them hypothesise that even though, dendritic spines could act as enclosed areas due to their mushroom-like morphology with a rather narrow neck, the biochemical effect tends to spread to neighbouring spines.\textsuperscript{201–203} Furthermore, even though the uncaging area of glutamate is quite small, diffusion of uncaged glutamate might have an effect on neighbouring spines in a time-delayed manner.

Figure 141: Visualisation of fluorescence change at the position of uncaging. Prior to uncaging, the fluorescence is constant. Within seconds after the uncaging laser pulse train started (indicated by a black bar), the fluorescence increases approx. three fold. After five minutes, the fluorescence drops to two-fold compared to background level.
Local miRNA Maturation

The number of pulses, before fluorescence signal increase starts, differs between cells. Other cells did not always show the effect of growing neighbouring spines. A more detailed view of the changes within the dendritic spines upon glutamate uncaging reveals that spine size changes rapidly within 60 s after glutamate uncaging has started. These changes in spine morphology correspond well to the increase in fluorescence due to Dicer-dependent probe processing.

![Glutamate uncaging (2 ms, 1 Hz)](image)

Figure 142: Glutamate uncaging triggers miRNA-181a1 maturation in the corresponding spine close to the uncaging point. Alexa488 staining (green, upper row) also reveals that this pre-miRNA processing is associated with a morphological change of the spine.

Regarding localised pre-miRNA processing within the dendritic spine itself, the following figure shows that there is hardly any probe processing within the shaft, which is close to the spine. Full fluorescence signal can be found in the region of the post-synaptic density close to the post-synaptic membrane. The fluorescence within the shaft corresponds to the background fluorescence for at least up to 60 s. Due to these results, the possibility of diffusion as the main reason for fluorescence increase could be excluded.
Figure 143: Pre-miRNA-181a1 probe processing in a single spine upon local glutamate uncaging. a) 5 s after glutamate uncaging, the fluorescence signal increases. After 25 s the fluorescence stays constant for up to 60 s (cf. part b). The maximal fluorescence is close to the membrane of the spine and might well correspond to the position of the PSD. There is hardly any pre-miRNA probe processing in the shaft of the spine.

Summary

In this study, a probe was successfully designed that is able to reflect Dicer activity by fluorescence signal increase. Upon Dicer-mediated processing of pre-miRNA-181a1, a fluorophore and a quencher are separated from each other, so that fluorescence can be visualised.

In contrast to published probes, this current probe corresponds to native pre-miRNAs as close as possible. Structural characteristics such as the 2-nt-overhang of the stem-loop structure are intact and no alternative cleavage sites are present.

Several fluorophore-quencher pairs were tested and even bulky modifications did not interfere with Dicer activity, as long as the two modified nucleotides were incorporated on different sides of the sequence.

Afterwards, it was proven by in vitro testing that the probe was generally working. The addition of RNase inhibitors in high amounts further proved the functionality of the probe design. Especially when neuronal cell lysate was used, the signal-to-noise ratio was convincing with a fluorescence increase of up to 10 compared to an undigested pre-miRNA probe.
Unfortunately, it was not possible to identify a well-performing Dicer-specific inhibitor, even though especially peptides, which mimic parts of the Dicer sequence, had been shown to work well in literature\textsuperscript{302}. Instead, RNA-binders such as the aminoglycoside antibiotic Neomycin worked well \textit{in vitro}. However, the necessary concentration of Neomycin was rather high. Additionally, there is no specific binding to pre-miRNA-181a1 or even pre-miRNAs in general. Due to this fact, undesired side effects during \textit{in vivo} experiments could not be excluded, because even protein biosynthesis might be impaired.

As an alternative biochemical proof of principle, neuronal ganglia of Dicer knock-out mice were used for control \textit{in vitro} experiments. In Dicer KO mice cell lysate hardly any probe processing was found, as it would be expected, if the probe is Dicer-specific and if unspecific RNase-mediated digestion can be excluded. In \textit{in vivo} experiments, it could be proven that Dicer is actually present in neuronal dendrites – a prerequisite for its local activity. Furthermore, the presence of native pre-miRNA-181a1 in dendritic spines was shown. It is known from literature that there is no general diffusion-dependent presence of pre-miRNAs everywhere in a neuron. On the one hand, there is a number of miRNAs that are tissue-specific\textsuperscript{327}. On the other hand, some miRNAs are also known to be exclusively somatically localised such as miRNA-134\textsuperscript{233} and – rather controversially discussed – miRNA-124\textsuperscript{226}.

While transfection methods of the probe failed, patch clamp technique – as a single cell method – worked well in delivering the probe to neurons. The highest fluorescent read-out was always found in the soma and proximal dendrites. Depolarisation of the neuron increased fluorescence signal, indicating enhanced Dicer-mediated pre-miRNA processing. Interestingly, the presence of the NMDAR inhibitor APV fully abolished fluorescence signal increase despite simultaneous depolarisation of the cell. Furthermore, the dendritic level of Dicer activity was even below basal levels.

These findings are strong evidence for an NMDAR- and, respectively, Ca\textsuperscript{2+}-dependent activation pathway of miRNA-181 in dendrites.

In a next step, the dendritic processing was visualised by local uncaging of glutamate. In these experiments, photoresponsive glutamate is released by a train of laser pulses. Upon uncaging of the neurotransmitter glutamate at either proximal or distal dendrites, Dicer-mediated processing at certain dendritic spines could be visualised. It was sometimes even possible to observe growth of the activated spine itself or its neighbour after a certain time point. These findings show that dendritically found mature miRNA does not necessarily derive from diffusion to the dendrites, but might well result from Dicer processing of dendritically localised, dormant pre-miRNA.

**Future Prospects**

Even though the present results are strong evidence for the hypothesised activity-dependent, local miRNA maturation in neuronal dendrites, further verification is needed. It clearly suggests itself to design an equivalent probe of an exclusively somatically localised miRNA as negative control. A probe like this might result in somatically fluorescence increase, when the whole neuron is depolarised. It cannot be excluded that fully processed, mature miRNA then diffuses to the dendrites. However, glutamate uncaging that is locally
restricted to the dendrites, as it has been discussed above, should not result in fluorescence increase or any visible change in spine size.

Possible candidates would be pre-miRNA-134, for which it has been proven not be present in dendrites by SCHRATT and his group.\textsuperscript{233} Apart from that, pre-miRNA-124 is controversially discussed. Some studies suggested presence of pre-miRNA-124 mainly in the soma. It was even used as non-dendritically localised control pre-miRNA.\textsuperscript{226,228,230} However, in 2014, Ho et al. detected pre-miRNA-124 in dendrites by \textit{in situ} hybridisation.\textsuperscript{320} Moreover, it seems to have an important role in neurite growth and elongation by targeting mRNA of ROCK1.\textsuperscript{329} Even the mRNA of the GTPase RhoG seems to be targeted by miRNA-124. RhoG plays a central role in neurite outgrowth by actin remodelling.\textsuperscript{330} These findings clearly suggest its presence in dendrites.

Another approach is the usage of tissue-specific pre-miRNA. GUO et al. screened a number of pre-miRNAs and – among others – found that pre-miRNA-449 is specific to testis.\textsuperscript{327} Candidates like that might also be suitable as negative control, because without a dendritically localised target, the pre-miRNA-449 is most likely not transported there.

First, the potential candidates and their exclusively somatical localisation should be verified by FISH experiments. Therefore, a complementary fluorescent probe against the respective loop sequence needs to be designed, as it has been done for pre-miRNA-181a1 for this study as well.

Secondly, it might be interesting to see, if the generated, fluorescently labelled mature miRNA-181 derivative is fully active. For this purpose, protein levels of the respective mRNA could be compared at a certain time point after probe delivery. For miRNA-181, targets would be GluA2\textsuperscript{291} and presumably CamKII. However, the bulky fluorescent TAMRA residue as well as the fact that the modification is attached at a DNA nucleotide might interfere with RISC loading and miRNA action in general. Notably, the modification is not in the region of the seed sequence, which needs to be perfectly complementary and is located at the 5’-end.

Furthermore, for experiments like that, delivered probe amounts must be chosen carefully. In the current study, the aim was to visualise the processing without any respect to the biochemical consequences and the long-term fate of the neuron. If this would be of interest, the delivered probe amount might need to be re-optimised. Excessive, unnatural amounts of pre-miRNA-181a1 and mature miRNA, respectively, might be critical for the cell.

To visualise newly synthesised proteins or changes in their expression levels, the so-called \textit{FUNCAT technique} (abbrev.: fluorescent non-canonical amino acid tagging) can be employed. In this method, cells are provided with the azide-bearing amino acid azidohomoalanine (AHA) as a methionine surrogate. Once AHA has been used during protein biosynthesis, the newly synthesised, azide-labelled proteins can then be tagged by alkyne-modified fluorophores in a CuAAC reaction.\textsuperscript{331,332}
Additionally, the NanoString technology could be used to estimate the number and the half-life of pri- and pre-miRNA-181a1, respectively.

The NanoString technology works with two different hybridisation probes per RNA sequence of interest. The capture probe is used for immobilisation, while the reporter probe is labelled with a number of fluorophores in a barcode-like structure. Like this, up to several hundred RNAs can be detected and counted simultaneously, because every RNA is characterised by its specific colour-coded barcode.

For future studies, it would also be interesting to know how the transport of dendritically targeted pre-miRNAs is mediated. For mRNAs, studies suggest the transport of dormant-state mRNA in granules of controversially discussed composition. For pre-miRNAs this has not yet been elucidated except for the fact that the loop sequence seems to play a zip-code-like role. Nothing is known about the detailed recognition sequences and – most likely – not all of the assisting proteins have been identified.
Experimental Part

Chemical Synthesis

Materials and Reagents

Commercially available reagents were mostly ordered fromSigma-Aldrich, Fluka, Acros, TCI, ChemGenes, and Carbosynth and were used without further purification. Regarding preparative normal phase chromatography, technical grade solvents were used as eluents. One exception was the purification of phosphoramidites that have been purified using pro analysis quality solvents.

All reactions were performed under argon atmosphere using dry solvents.

Thin Layer Chromatography

Thin layer chromatography (TLC) was used to monitor reaction progress. TLC was performed on aluminum plates coated with silica gel 60 F254 labelled with a fluorescent indicator for detection using 254 nm UV light (Merck). For staining, the following solutions were used, when necessary:

- Anisaldehyde solution (10 mL p-anisaldehyde, 5 mL glacial acetic acid, 420 mL ethanol und 15 mL concentrated sulfuric acid),
- Ninhydrin solution (0.6 g ninhydrin, 2 mL glacial acid, 13 mL water und 420 mL n-butanol).

Preparative Normal Phase Chromatography

After reaction, the raw products were purified using flash chromatography in glass columns under air pressure. As solid phase, silica gel 60 by Machery-Nagel GmbH&Co-Kg was used with a particle size of 0.04-0.063 µm.

Analytical Devices

NMR-Spectroscopy

NMR measurements were performed using one of the following spectrometers:

- Bruker AM 250 (250 MHz 1H-NMR)
- Bruker AV 300 (300 MHz 1H-NMR, 121.5 MHz 31P-NMR)
- Bruker AV 400 (400 MHz 1H-NMR, 162 MHz 31P-NMR)
- Bruker DPX 250 (250 MHz 1H-NMR)

For easier signal correlation, additional two-dimensional 1H-1H-COSY spectra were measured. All measurements were performed at 300 K. The chemical shifts are correlated to the respective solvent: CDCl3 (1H, δ = 7.26), DMSO-d6 (1H, δ = 2.50) und Aceton-d6 (1H, δ = 2.05) in ppm. The shift of 31P-NMR signals for phosphoramidite analysis was measured against an external standard of phosphoric acid (85%).
Experimental Part

**Mass Spectrometry**
Mass analysis was performed on a Bruker micro-TOF-Q. For high-resolution mass spectrometer, MALDI LTQ Orbitrap XL (Thermo Fisher Scientific) was used with α-cyano-4-hydroxy cinnamic acid or 2,5-dihydroxy benzoic acid as matrix. Oligonucleotides were characterised via mass spectroscopy (microTOF-Q by Bruker Corporation, negative mode).

**Synthesis Procedures**

**3’-5’-Di-O-(tert-butylidimethylsilyl)-2’-desoxyguanosine**

2’-Desoxyguanosine (10.0 g, 37.4 mmol, 1 equiv) and imidazole (16.8 g, 247.3 mmol, 6.6 equiv) were dissolved in 100 mL DMF under inert gas. Subsequently, tBDMS-Cl (22.6 g, 149.6 mmol, 4 equiv) was added and the reaction mixture was stirred at room temperature overnight. Then, the reaction was quenched by adding 50 mL ethanol. The solvent was removed in vacuo. The residue was again solved in CH$_3$Cl and extracted with 5% (w/v) citric acid and aqueous NaHCO$_3$ (10%, (w/v)). Afterwards, the organic layer was dried over MgSO$_4$ and the solvent was again removed to yield a white powder (18.92 g, 38.21 mmol, quantitative).$^{335}$

R$_f$ = 0.54 (DCM:MeOH 90:10 (v/v))

$^1$H NMR (400 MHz, DMSO-d$_6$): δ = 0.10-0.08 (12H, 2x Si(CH$_3$)$_2$) 0.90-0.88 (18H, 2x SiC(CH$_3$)$_3$), 2.62-2.66 (m, 2H, H-2’), 3.69-3.79 (m, 2H, H-5’), 3.87-3.93 (m, 1H, H-4’), 4.40-4.56 (m, 1H, H-3’), 5.79 (t, 1H, J = 6.5 Hz, H-1’), 6.47 (bs, 2H, NH$_2$), 7.89 (s, 1H, H-8), 10.97 (s, 1H, NH) ppm.

**MS:** calculated for C$_{22}$H$_{41}$N$_5$O$_4$Si$_2$: 495.27, measured: 497.41.
**3’-5´-Di-O-(tert-butyldimethylsilyl)-N²-(4-isopropylphenoxyacetyl)-2´-desoxyguanosine**

The tBDMS-protected nucleoside (10 g, 20.2 mmol, 1.0 equiv) was dissolved in 250 mL pyridine and the solution was cooled to 0 °C. Subsequently, the previously synthesised 4-iPr-C₆H₄-OCH₂COCl (4.29 g, 30.3 mmol, 1.5 equiv) was added dropwise and the resulting solution was stirred for 4 h within a thawing ice bath. After quenching with methanol (150 mL) at 0 °C, the solvent was removed in vacuo. Column chromatography (cyclohexane:acetone = 3:1 → 2:1) afforded the above shown nucleoside as yellowish solid foam (8.47 g, 12.61 mmol, 63%).

Rf = 0.32 (DCM:MeOH=95:5 (v/v))

1H NMR (400 MHz, DMSO-d₆): δ = 0.10-0.08 (12H, 2x Si(CH₃)₂) 0.90-0.88 (18H, 2x Si(CH₃)₃), 1.19 (d, 6H, J = 6.9 Hz, iPrPac CH₃), 2.36-2.42 (m, 2H, H-2'), 2.82-2.90 (m, 1H, iPrPac CH), 3.68-3.78 (m, 2H, H-5'), 3.85-3.92 (m, 1H, H-4'), 4.44-4.56 (m, 1H, H-3'), 4.68 (s, 2H, iPrPac CH₂O), 5.89 (t, J = 6.5 Hz, 1H, H-1'), 6.93 (d, 2H, d, J = 8.6 Hz, iPrPac CH₂H), 7.18 (d, 2H, J= 8.6 Hz, iPrPac CH₂H), 7.97 (s, 1H, H-8), 9.19 (bs, 1H, NH), 11.75 (bs, 1H, NH) ppm

MS: calculated for C₃₃H₅₃N₅O₆Si₂: 671.35, measured: 672.57.

**3’-5´-Bis-O-(tert-butyldimethylsilyl)-N²-(4-sopropylphenoxyacetyl)-O⁶-[2-(2-nitrophenyl)propyl]-2´-desoxyguanosine**

The previous product (8.0 g, 11.9 mmol, 1.0 equiv), 2-(2-nitrophenyl)-propan-1-ol (NPP-OH, 3.3 g, 17.9 mmol, 1.5 equiv) and PPh₃ (4.7 g, 17.9 mmol, 1.5 equiv) were dissolved in 250 mL THF. Afterwards, a solution of diethylazodicarboxylate (DEAD, 40% in toluene, 8.2 mL, 17.8 mmol, 1.5 equiv) was added under inert gas. After 2 h at room temperature, the solution was diluted with DCM, extracted, and dried over MgSO₄. After removal of the solvents in vacuo, the raw product was purified by column chromatography (cyclohexane:ethyl acetate = 5:1 → 1:1). A yellowish solid foam was afforded as diastereomeric mixture (4.5 g, 5.4 mmol, 45%).

Rf = 0.29 (CH:EE =1:1 (v/v))
**Experimental Part**

\textbf{1H NMR} (400 MHz, DMSO-\textit{d}_{6}): \(\delta = 0.10-0.09\) (12H, 2x Si(CH\textsubscript{3})\textsubscript{2}), 0.92-0.80 (18H, 2x SiC(CH\textsubscript{3})\textsubscript{3}), 1.19 (d, 6H, \(J = 6.9\) Hz, iPr CH\textsubscript{3}), 1.52 (d, 3H, \(J = 6.8\) Hz, NPP CH\textsubscript{3}), 2.34-2.44 (m, 2H, H-2'), 2.80-2.93 (m, 1H, iPrPac CH), 3.67-3.78 (m, 2H, H-5'), 3.89-4.06 (m, 2H, H-4', aliphatic NPP-CH) 4.44-4.86 (m, 5H, CH\textsubscript{2}O NPP, CH\textsubscript{2}O iPrPac, H-3'), 5.88 (t, \(J = 5.4\) Hz, 1H, H-1'), 6.93 (d, 2H, \(J = 8.6\) Hz, iPrPac CH\textsubscript{ar} H), 7.22 (d, 2H, \(J = 8.6\) Hz, iPrPac CH\textsubscript{ar}), 7.32-7.37 (m, 1H, CH\textsubscript{ar} H-4 NPP), 7.51-7.57 (m, 1H, CH\textsubscript{ar} H-5 NPP), 7.58-7.72 (m, 2H, CH\textsubscript{ar} H-6, H-3 NPP), 7.95 (s, 1H, H-8), 9.56 (bs, 1H, NH) ppm

**MS:** calculated for C\textsubscript{42}H\textsubscript{62}N\textsubscript{6}O\textsubscript{8}Si\textsubscript{2}: 834.41, measured: 833.42.

\textit{N\textsuperscript{2}-(4-isopropylphenoxyacetyl)-O\textsuperscript{6}-(2-(2-nitrophenyl)propyl)-2'-desoxyguanosine}

\[
\text{HO} \quad \text{O} \quad \text{NPrPac} \\
\text{OH} \\
\text{CH} \quad \text{ar} \\
\text{NO} \quad \text{N}
\]

The previous product (4.4 g, 5.3 mmol, 1 equiv) was dissolved in THF (100 mL) at 0 °C and acetic acid glacial (1.8 mL, 31.7 mmol, 6 equiv) and tetra-butylammonium fluoride (1M in THF, 15.8 mL, 15.8 mmol, 3 equiv) were added. The reaction mixture was stirred at room temperature overnight. Evaporation of the solvent and column chromatography (DCM:MeOH = 100:0 \(\rightarrow\) 90:10) afforded a brown foam (3.0 g, 5.0 mmol, 95%).

\(R_f = 0.32\) (DCM:MeOH=9:1 (v/v))

\textbf{1H NMR} (400 MHz, DMSO-\textit{d}_{6}): \(\delta = 1.24\) (d, 6H, \(J = 6.9\) Hz, iPr CH\textsubscript{3}), 1.49 (d, 3H, \(J = 6.9\) Hz, NPP CH\textsubscript{3}), 2.34-2.45 (m, 1H, H-2'), 2.88-2.94 (m, 1H, \(J = 6.9\) Hz, iPrPac CH), 2.94-3.05 (m, 1H, H-2'), 3.72-4.10 (m, 3H, H-5', H-4'), 4.12-4.15 (m, 1H, NPP CH\textsubscript{2}), 4.46-4.84 (m, 4H, NPP CH\textsubscript{2}, iPrPac CH\textsubscript{2}), 4.94-4.98 (m, 1H, H-3'), 5.10-5.16 (m, 1H, 3'-OH), 5.21-5.25 (m, 1H, 5'-OH), 5.98 (t, 1H, \(J = 5.4\) Hz, H-1'), 6.96 (d, 2H, \(J = 8.7\) Hz, CH\textsubscript{ar} iPrPac), 7.18 (d, 2H, \(J = 8.6\) Hz, CH\textsubscript{ar} iPrPac), 7.30-7.36 (m, 1H, CH\textsubscript{ar} H-4 NPP), 7.51-7.56 (m, 1H, CH\textsubscript{ar} H-5 NPP), 7.59-7.72 (m, 2H, CH\textsubscript{ar} H-6, H-3 NPP), 7.99 (s, 1H, CH\textsubscript{ar} H-8), 9.54 (bs, 1H, NH) ppm

**MS:** calculated for C\textsubscript{30}H\textsubscript{34}N\textsubscript{6}O\textsubscript{8}: 606.25, measured: 606.24.
5'-O-(4,4'-Dimethoxytrityl)-N²-(4-isopropylphenoxyacetyl)-O⁶-[2-(2-nitrophenyl)-propyl]-2'-desoxyguanosine

In the next step, the deprotected nucleoside (3.0 g, 4.9 mmol, 1 equiv) was dissolved in 100 mL pyridine, dimethoxytrityl chloride (2.1 g, 5.9 mmol, 1.2 equiv) was added, and the mixture was stirred for 48 h at room temperature. Afterwards, 100 mL MeOH was added, stirred for another 30 min, and the solvents were removed in vacuo. The raw product was purified via column chromatography (DCM:MeOH = 100:0 → 95:5). Notably, the column was washed with 1% (v/v) triethylamine first to neutralise the silica gel. After purification, a bright yellow foam was afforded as diastereomeric mixture (3.46 g, 3.5 mmol, 77%).

\[ R_f = 0.29 \ (9:1 \ DCM:MeOH \ (v/v)) \]

\[^{1}H\text{ NMR}\] (400 MHz, DMSO-\(d_6\)): \(\delta = 1.24 \ (d, 6H, J = 6.9 \ Hz, \text{iPr CH}_3), 1.50 \ (d, 3H, J = 6.9 \ Hz, \text{NPP CH}_3), 2.48-2.68 \ (m, 2H, H-2'), 2.88-2.94 \ (m, 1H, J = 6.9 \ Hz, \text{iPrPac CH}), 3.28-3.44 \ (m, 2H, H-5'), 3.68-3.76 \ (m, 6H, DMTr methoxy), 3.99-4.14 \ (m, 2H, \text{NPP CH}), 4.22-4.28 \ (m, 1H, H-4'), 4.53-4.87 \ (m, 5H, 3' H, \text{NPP CH}, \text{NPP CH}_3, \text{iPrPac CH}), 6.63 \ (t, 1H, J = 6.0 \ Hz, H-1'), 6.72-6.84 \ (m, 4H, \text{CH} \_\text{ar}, \text{DMTr-OMe}), 6.95 \ (d, 2H, J = 8.7 \ Hz, \text{CH} \_\text{ar}, \text{iPrPac}), 7.12-7.42 \ (m, 12H, \text{CH} \_\text{ar}, \text{iPrPac}, \text{DMTr}, \text{NPP}), 7.52-7.57 \ (m, 1H, \text{H-5 NPP}), 7.60-7.66 \ (m, 1H, \text{CH} \_\text{ar}, \text{H-6 NPP}), 7.70-7.75 \ (m, 1H, \text{CH} \_\text{ar}, \text{H-3 NPP}), 8.14 \ (s, 1H, \text{CH} \_\text{ar}, \text{H-8}), 9.24 \ (bs, 1H, \text{NH}) ppm

\[^{13}C\text{ MS}\] calculated for \(C_{51}H_{52}N_6O_{10}\): 908.36, measured: 909.38.
$5\text{'}-O-(4,4\text{'}-\text{Dimethoxytrityl})-N^\text{2}\text{-}(4\text{-isopropylphenoxyacetyl})-O^\text{6}\text{-}[2\text{-}(2\text{-nitrophenyl})\text{-propyl}]-2\text{-desoxyguanosin-3\text{'}-O}\text{-}(2\text{-cyanoethyl-N,N-diisopropyl})\text{-phosphoramidite}$

The DMTr-protected nucleoside (0.5 g, 0.6 mmol, 1 equiv) was dissolved in 50 mL THF and 0.33 mL NEtiPr$_2$ (1.9 mmol, 3.5 equiv) was added. After 15 min at room temperature, 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (0.25 mL, 1.1 mmol, 2 equiv) was added. The solution was stirred for 120 min at room temperature. Subsequently, aqueous NaHCO$_3$ (5% (w/v)) was added and the reaction mixture was extracted into ethyl acetate. The organic extracts were washed with brine and dried over MgSO$_4$. After solvent removal, column chromatography was conducted (cyclohexane:ethyl acetate = 4:1). Again the column was flushed with 1% (v/v) NEt$_3$ first for neutralisation. A yellowish foam was afforded (0.4 g, 0.35 mmol, 63%).$^{259}$ $R_f = 0.56$ (CH:EE = 1:4 (v/v))

$^1$H NMR (400 MHz, DMSO-d$_6$): $\delta = 1.04\text{-}1.22$ (m, 12H, NC(CH$_3$)$_2$), 1.25 (d, 6H, $J = 6.5$ Hz, CH$_3$iPrPac), 1.51 (d, 3H, $J = 6.8$ Hz, NPP CH$_3$), 2.45 (t, 1H, $J = 6.40$, CEO), 2.48-2.70 (m, 2H, H-2'), 2.62 (t, 1H, $J = 6.5$ Hz, CEO), 2.89-2.94 (m, 1H, iprPac CH), 3.27-3.42 (m, 2H, H-5'), 3.53-3.64 (m, 2H, NCH), 3.64-3.89 (m, 2H, DMTr CH$_3$), 3.72-3.80 (m, 6H, DMTr CH$_3$), 3.95-4.13 (m, 1H, NPP CH), 4.24-4.31 (m, 1H, H-4'), 4.66-4.86 (m, 5H, 3'-H, NPP CH$_2$O, iPrPac CH$_2$O), 6.47 (t, 1H, $J = 6.4$ Hz, H-1'), 6.74-6.84 (m, 4H, CH$_{ar}$ DMTr-OMe), 6.96 (d, 2H, $J = 8.4$ Hz, CH$_{ar}$ iPrPac), 7.14-7.44 (m, 12H, 2 CH$_{ar}$ iPrPac, DMTr, NPP), 7.48-7.56 (m, 1H, CH$_{ar}$ H-5 NPP), 7.61-7.65 (m, 1H, CH$_{ar}$ H-6 NPP), 7.71-7.75 (m, 1H, CH$_{ar}$ H-3 NPP), 8.11 (s, 1H, CH$_{ar}$ H-8), 9.12 (bs, 1H, NH) ppm

$^{31}$P NMR (121.5 MHz, DMSO-d$_6$): $\delta = 149.6$

MS: calculated for C$_{60}$H$_{69}$N$_8$O$_{11}$P: 1108.48, measured: 1109.65.

**Further synthesis procedures**

Synthesis procedures of quantum dots and caged QCy7 derivatives can be found in the Bachelor and Master Theses of SVENJA R. BIRON, JULIANA ERSCH, and ALEXANDRA GRESIKA.
General Methods

Oligonucleotide Synthesis

**Feedback Loop Tracking and Molecular Beacon Project**

Molecular beacons were synthesised on an Applied Biosystems Model (ABI) 392 synthesiser using 2'-O-methyl-RNA phosphoramidites purchased from Link Technologies Ltd. All phosphoramidites were employed according to their recommended coupling protocols. Modifications such as dye phosphoramidites were mostly purchased from Link Technologies Ltd. or ChemGenes Corporation and introduced with a 15 min coupling time. Cy3 phosphoramidite was ordered from GE Healthcare Limited and employed with a 6 min coupling time. Desoxyguanosine phosphoramidite modified with NPP (2-(2-nitrophenyl)-propyl) as photocleavable group was synthesised as described previously and used for solid phase synthesis with a 6 min coupling time. The synthesis was performed with solid support (200 nmol scale) purchased from Link Technologies Ltd.

After synthesis, most oligonucleotides were mostly cleaved from the solid support and deprotected with fresh ammonia (32% (v/v)) solution at 65 °C for two hours.

The RNA target used for the *in vitro* testing of the respective Molecular Beacons was ordered from IBA GmbH, Göttingen, Germany.

**Pre-miRNA Project**

RNA oligonucleotides were also synthesised on an ABI 392 synthesiser using 2'-tBDMS-protected RNA phosphoramidites purchased from Sigma Aldrich. Modifications such as internal amino modifier, dye-labelled dT phosphoramidites, or chemical phosphorylation reagents for the introduction of 5'-terminal phosphate moiety were exclusively purchased from Link Technologies Ltd. During synthesis, all phosphoramidites were coupled with a 15 min coupling time. The synthesis was performed on 1 µmol CPG support purchased from Link Technologies as well. The DMTr-OFF mode was chosen.

After synthesis, the RNA oligonucleotides were cleaved from the solid support by a mixture of fresh 32% aq. ammonia and ethanol (3:1 (v/v)) for 24 h at room temperature. One exception of this procedure is the preparation of oligonucleotides that contain TAMRA as fluorophore. These oligonucleotides have to be deprotected using t-butylamine/water (1:3) for 6 hours at 60 °C. Afterwards the 2'-tBDMS PG is removed by a mixture of N-methylpyrrolidion (NMP, 300 µL), triethylamin (TEA, 150 µL), and triethylamin-trihydrofluorid (TEA*3HF, 200 µL) for 90 min at 60 °C.

To remove fluoride prior to HPLC purification, the oligonucleotide was precipitated in 1.5 mL 1-buthanol at -80 °C for at least 4 h.
Experimental Part

Oligonucleotide Purification

**Molecular Beacons**
After synthesis and deprotection, oligonucleotides were purified via reversed-phase HPLC. Various C\textsubscript{18} reversed phase columns were used with a gradient of 0.1 M TEAA (triethylammonium acetate, pH 7) and acetonitrile. The percentage of acetonitrile was increased from 0% to 47% within 50 min. The column was heated to 55 °C.

**Pre-miRNA Project**
After synthesis, the RNA oligonucleotide was purified via anion exchange (AE) HPLC (80 °C) with increasing amounts of LiCl (1 M, 0%-100%). It should be noted, that all HPLC solutions and buffers had been treated with diethylpyrocarbonate (DEPC, 1:1000 dilution) in advance to inactivate RNases. After AE-HPLC, the oligonucleotides were further purified via RP-HPLC to remove excessive salt. C\textsubscript{12} or C\textsubscript{18} reversed phase columns were used with 0.1 M TEAA and acetonitrile. The percentage of acetonitrile was increased from 5% to 55% within 40 min.

**Concentration Determination**
The concentration of oligonucleotides was determined at the NanoDrop 200 UV/Vis spectrometer by Thermo. Even though the spectrometer is able to calculate oligonucleotide concentration, these values are only reliable for long DNA/RNA strands. In this study, the oligonucleotides are usually not long enough to approximate an even distribution of all bases within the strand. Additionally, modifications such as dyes also absorb at 260 nm due to their aromatic structure and must be included into the calculation. For this reason the NanoDrop was exclusively used for measuring the absorption at 260 nm (A\textsubscript{260}). Even though the NanoDrop is supposed to determine the concentration and absorption linearly over a wide range, all samples were diluted to a absorption range below 100. The A\textsubscript{260} is given for a theoretical path length of 1 cm.

The extinction coefficient $\varepsilon$ at 260 nm is known for all nucleobases and most dyes. Modifications such as photocleavable groups or linkers were ignored, since they either do not influence $\varepsilon$ or show an insignificant influence. The concentration can then be calculated according to the *Beer-Lambert law*:

$$c \frac{mol}{L} = \frac{A_{260}}{1cm \times \sum \varepsilon}$$

**Post-synthetic NHS Ester Labelling**
The oligonucleotide, which was supposed to be labelled, was concentrated to approx. 100 µM. The pH was adjusted to pH 8.3-8.5 with 10% NaOH (w/v) and 0.2 M NaHCO\textsubscript{3}. Afterwards, two to three equivalents of NHS ester in relation to free amino groups within the oligonucleotide were added. The master mix concentration of the NHS-ester was set to 50-200 mg/mL with anhydrous DMSO or DMF according to manufacturer’s protocol. The reaction mixture was kept at room temperature for 90 min.

Afterwards, the oligonucleotide can be precipitated using ethanol prior to purification. Alternatively, the reaction mixture can directly be purified via RP-HPLC with 0.1 M TEAA.
buffer and acetonitrile. The solvent gradients were chosen according to the labelled oligonucleotide, as described before.

**Post-synthetic Maleimide Labelling**
The thiol-modified oligonucleotide was dissolved in 1x PBS buffer (pH 7.0). Afterwards the solution was degassed with argon. An excess (1.1 eq) TCEP (tris-carboxyethylphosphine) reagent was added to reduce dithiol bonds and the reaction mixture was kept for 20 min at room temperature. The maleimide-functionalised modification was meanwhile dissolved in dry DMSO, the concentration was set to 50-200 µM. A high excess (up to 20 eq) of the maleimide was then added to the oligonucleotide. The reaction mixture was once again flushed with argon. Afterwards the reaction was kept at room temperature overnight. Subsequently, the successfully labelled oligonucleotide could be purified via RP-HPLC.

**Post-synthetic CuAAC Labelling**
The concentration of the alkyne-modified oligonucleotide was set to 100 µM with RNase-free water. 1 M TEAA buffer was added to a final concentration of 0.2 M (pH 7.0). The solution was degassed by bubbling argon through the solution. The azide-functionalised dye was dissolved in DMF:MeOH (1:1 (v/v), 10 mM stock concentration) and 1.5 equivalents were added to the oligonucleotide-containing solution. Ascorbic acid was added to a final concentration of 0.5 mM. Importantly, the stock solution of ascorbic acid had been prepared freshly. The reaction was degassed again for approx. 30 s. Subsequently, the copper(II)-TBTA (final concentration 25 mM in 55% DMSO (w/v)) was added and the reaction mixture was kept overnight at 45 °C.
After the reaction had finished, the oligonucleotide was either purified via RP-HPLC directly or first precipitated with acetone.

**Fluorescence Measurement**
Fluorescence was measured with a plate reader (Tecan Infinite M200 Pro Plate Reader) using black 96-well plates with a non-binding surface and a flat bottom by Corning. The gain of the plate reader was set to optimal. Like this, measured values cannot be compared directly, but positive or negative controls, respectively, were always included into measurements. The samples were always measured as triplets and mean values were taken for calculations. The number of flashes was always set to 25 per measurement. The gap width was 20 nm for excitation light and 9 nm for emission light. As a result, fluorescence was measured at the maximal excitation and emission wavelength, as long the distance was at least 30 nm. All measurements were conducted in 1x PBS buffer pH 7.4.
Illumination Experiments

For illumination/derotation experiments, three different instruments with 365 nm illumination wavelength were used.

<table>
<thead>
<tr>
<th>Group internal name</th>
<th>Conduction current</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-LED 365-250-SMD (Multi LED)</td>
<td>500 mA</td>
<td>250 mW</td>
</tr>
<tr>
<td>UV-LED 365 100 (preparative illumination)</td>
<td>500 mA</td>
<td>7.04 W</td>
</tr>
<tr>
<td>UV-LED (top, middle, bottom illumination)</td>
<td>500 mA</td>
<td>130-154 mW</td>
</tr>
</tbody>
</table>

The amount of sample that was supposed to be illuminated was dissolved in 1x PBS buffer (pH 7.4) to ensure a constant pH value under physiological conditions during illumination. The samples were illuminated for varying time periods. The progress of the photoresponsive reaction was checked via RP-HPLC or fluorescence measurements, respectively.

In vitro Light-Activation of Molecular Beacons

For light-induced removal of the NPP-group or cleavage of the commercially available photocleavable linker, the Molecular Beacon was irradiated for up to 3 min with 365 nm and 250 mW power using the LED “UVLED-365-250-SMD” from Roithner Lasertechnik. The irradiation experiments were always conducted in PBS buffer (1x, pH 7.4).

Precipitation of Oligonucleotides

As TEAA is known to be cytotoxic, it is strongly recommended to remove residual amounts of TEAA after RP-HPLC. For this reason, oligonucleotides that were supposed to be tested in cells or cell lysates were precipitated three times with ethanol. For this reason, the concentration of the oligonucleotide was set to an absorption (260 nm) of 100 with 0.3 M sodium acetate (pH 5.2). Afterwards, three volumes of ethanol were added, the mixture was cooled to -20 °C for 30 min and finally centrifuged for 30 min at full speed. The supernatant was removed and the procedure was repeated two more times. After the final precipitation, the pellet was dissolved in RNase-free water or 1x PBS buffer.

Phenolic RNA Extraction

The phenolic extraction is a liquid extraction method to isolate DNA and RNA out of a reaction mixture or cell lysate. The partitioning of DNA and RNA between the organic phase and the aqueous one is determined by the pH of the phenolic solution. At the beginning, the pH is kept in the range between 7 and 8. At this pH, the phosphate backbone of both DNA and RNA are negatively charged and consequently both can be found in the aqueous layer. DNA can then be removed from the aqueous layer, as the pH is decreased to pH 4.5. At this acidic pH, most proteins and small DNA fragments (<10 kb) fractionate into the organic layer and large DNA fragments and some proteins remain at the interphase between the organic and aqueous phases, because the phosphate backbone of DNA is protonated more easily in comparison to RNA.336–338
An equivalent volume of aqueous sample and a mixture of phenol-TE buffer (pH 4.5), chloroform, and isoamyl alcohol (125:24:1, (v/v/v)) are mixed, vortexed for one minute and subsequently centrifuged at top speed for 5 minutes. Afterwards, the upper aqueous layer is transferred into a new tube and a mixture of chloroform:isoamyl alcohol (25:1, (v/v)) is added. The mixture is again vortexed and centrifuged. Afterwards, the RNA is precipitated by sodium acetate (0.3 M final concentration, pH 5.2) and ethanol, as it is described above.

Surface Modification of Quantum Dots
800 µL of the commercially available Lumidot CdSe/ZnS dispersion in Toluol (800 µL) was mixed with approx. 1 mL of 3-mercaptopropionic acid and slightly shaken o/n at RT. Afterwards, 80 µL 1 M NaOH was added, the aqueous layer was removed and mixed with acetone (50 µL). QDs were isolated by centrifugation (10 min, 1500/min). Remaining QDs were precipitated from the aqueous layer by subsequent addition of acetone (3x, 20 µL). After precipitation, the surface-functionalised QDs were dissolved in PBS buffer and concentration was approximated with the known molar extinction coefficient.

Biochemical & Molecular Biological Methods
Concerning this part, it should be noted that experiments that involve the usage of cells – except cell lysates – or microscopy techniques were conducted by the previously mentioned collaborating research groups.

Preparation and Microinjection of Chironomus tentans Salivary Glands
Cultivation of the dipteran C. tentans, dissection of salivary gland and microinjection of oligonucleotides were performed, as described in literature. Dissected glands were transferred onto poly-L-Lysine coated cover slips for microscopic observation and were incubated in PBS buffer during microinjection and confocal imaging.

Quantification of Molecular Beacon Activation and Fluorescence Yield
The relative intensity contributions of different MB designs within a cell were separated using spectrally resolved imaging with a LSM 510 META (Carl Zeiss Microscopy GmbH) equipped with a C-Apochromat 40X/1.2 W objective lens. In all experiments a HeNe laser emitting at 543 nm was used for fluorescence excitation. Fluorescence was separated from the excitation light using a HFT488/543 dichroic mirror, and detected using the META detector of the LSM. The collected fluorescence was split into 14 about 10 nm wide channels with their central wavelengths ranging from 558 nm to 697 nm.

DNase I Digestion of DNA Probes.
For digestion tests DNA beacons MB1a’ and MB4’ were synthesised, as described above. Deoxyribonuclease I (Amplification grade, Sigma Aldrich) was used. The reaction was
Experimental Part

carried out according to manufacturer’s protocol and with the provided reaction buffer (50 pmol MB sample, 0.1 μL of the provided DNase solution, 37 °C). Directly after addition of the enzyme the fluorescence was measured with a Tecan plate reader Infinite 200 PRO, as described above.

**Enzymatic Ligation**
Equivalent amounts of the two RNA strands as well as the splint DNA were used. The volume was adjusted to 294 μL by the addition of water. Afterwards, the sample was heated to 90 °C for 3 min and subsequently cooled to room temperature for 20 min. 40 μL of 10X ligation buffer and 40 μL of PEG 4,000 solution were added. Both solutions were usually provided by the manufacturer. 0.25 μL of T4 DNA ligase in storage solution and another 19.75 μL of water were added. The reaction mixture was incubated at 33 °C for 2 h. Finally, 3 μL of T4 RNA ligase (in storage solution diluted with 3 μL water) were added and the reaction is incubated for another 2.5 h at 33 °C.

**Enzymatic Digestion Using Recombinant Enzymes**
Varying amounts of pre-miRNA probes were incubated with different types of commercially available Dicer enzymes. The enzymes were applied according to manufacturer’s protocol. Usually, the probe was mixed with the according reaction buffer. Additives such as ATP (1 mM final concentration) or MgCl₂ were added in the case, if they had not been included into the buffer by the manufacturer. It must be noted, that especially ATP solutions have to be prepared freshly. The reaction times were chosen according to the respective enzyme protocol and differed between 2 h (TurboDicer, Genlantis) to 12-18 h (PowerCut Dicer, Finnzymes and Dicer enzyme kit by Genlantis). The incubation was always performed at 37 °C. Afterwards, reaction success was monitored by fluorescence measurement or polyacrylamide gel analysis. Digestion reactions with enzymes from Genlantis can be stopped using the provided Dicer stop solution. The content of reaction buffers and stop solutions were not disclosed by the manufacturer.

**In vitro Cleavage Assay with Neuronal Cell Lysate**
1 million hippocampal rat neurons (25-30 DIV) were lysed in 1 mL PBS using cell scrapers and pressed through syringes several times to disrupt them. Excessive cell fragments were removed by centrifugation. Varying amounts of pre-miRNA probe were incubated at 37 °C for 24-48 h. The reaction was conducted in Dicer reaction buffer provided by Finnzymes or 1X PBS buffer. The reaction was monitored regularly by fluorescence measurements. For some studies RNasin® Plus RNase inhibitor purchased from promega was added. The amounts of RNase inhibitor varied, mostly a dilution of 1:100 was used. It should be noted that the lysate was performed freshly directly prior to the digestion experiment. It was observed that digestion efficiency decreased to 30% within two days, even when stored at -80 °C.

**LipofectAmine 2000 Transfection**
According to manufacturer's protocol, cells need to be 70-90% confluent at transfection. The Lipofectamine reagent is divided into four equal amounts depending on cell culture well size
and diluted with Opti-MEM Medium. The DNA/RNA that is supposed to be transfected is diluted in Opti-MEM Medium. The optimal amount for 1,000,000 cells would be 14 μg total DNA/RNA. Afterwards the diluted DNA/RNA is added to the diluted Lipofectamine Reagent in a 1:1 ratio. The mixture is incubated for 5 min at room temperature. The DNA-lipid complex is then added to the cells and incubated at 37 °C for 2 h to 3 days before transfected cells can be analysed.

**Magnetofectamine Transfection**

The DNA/RNA is first diluted in an appropriate amount of Opti-MEM Medium without serum. In parallel, Lipofectamine is also diluted the appropriate amount in Opti-MEM Medium. Afterwards, the DNA/RNA mixture is added to the Lipofectamine reaction mixture, mixed gently and incubated 5 min at room temperature.

1 μL CombiMag is used per 1 μg DNA or 40 pmol DNA/RNA, respectively. The lipid-DNA/RNA complex is immediately added to the CombiMag reagent. If less than 1 μL is used, it can be diluted with deionised water. The mixture is incubated for 20 min at room temperature.

The culture plate to be transfected is then placed onto the magnetic plate and the complex reaction mixture is added to the wells and incubated for another 5 min onto the magnetic plate. The transfection mixture is incubated for 30 min at 37 °C. After that time, the magnetic plate can be removed.
Appendix

Oligonucleotide Sequences

Multiple Labelled Oligonucleotides (2’-OMe-RNA)

Table 8: Oligonucleotides for feedback tracking study.
X= dT\(^{\text{10M2}}\)
Y= NH\(_2\)-\((\text{CH}_2)\)\(_6\)-linker

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<th>Name</th>
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<tr>
<td>28S rRNA oligo</td>
<td>5'-Y-CAU UCG AAU AUU UGC XAC UAC CAC CAA GAU CUG-Y-3'</td>
</tr>
<tr>
<td>BR2.1 3x oligo</td>
<td>5'-Y-CUU GGC XUG CXG UGU XUG CXU GGX UUG C-Y-3'</td>
</tr>
<tr>
<td>BR2.1 1x oligo</td>
<td>5'-Y-ACU UGG CUU GCU GUG UUU GCU UGG UUU GCU-3'</td>
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</tbody>
</table>
Molecular Beacon Sequences

### Table 9: Oligonucleotides for MB study.

**Loop sequence**
- Z = dG<sup>pp</sup>
- PCL - photocleavable linker
- Fluo – Fluorescein

<table>
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<th>Name</th>
<th>Sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
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<td><strong>BR2.1 target RNA</strong></td>
<td>GAA CGG AAC GAC ACA AAC GAA CCA AAC G (RNA)</td>
</tr>
<tr>
<td><strong>BR2.1 pos. ctrl. MB</strong></td>
<td>Cy3-CAC GAC UUG GCU UGC UGU GUU UGC UGG GUU UGC UG-BHQ2 (2′OMe-RNA/DNA)</td>
</tr>
<tr>
<td>Loop caged MB 1a</td>
<td>Cy3-CAC GAC UUZ GCU UZC UZU ZUU UZC UUZ GUU UZC UG-BHQ2 (2′OMe-RNA/DNA)</td>
</tr>
<tr>
<td>Loop caged MB 1b</td>
<td>Cy3-CAC GAC TTZ GCT TGC TZZ GTT TZZ TGC TGT TG-BHQ2 (DNA)</td>
</tr>
<tr>
<td>Loop caged MB 1c</td>
<td>Cy3-CAC GAC TTG GCT TGT ZTG TGT TGC TCG TG-BHQ2 (DNA)</td>
</tr>
<tr>
<td>Caged Q-Fluorescein MB2</td>
<td>Dabcyl-PCL-Fluo-CAC GAC UUZ GCU UZC UZU ZUU UZC UUZ GUU UZC UG-BHQ2 (2′OMe-RNA)</td>
</tr>
<tr>
<td>Caged Q-Fluorescein MB3</td>
<td>Dabcyl-PCL-Fluo-CAC GAC UUZ GCU UZC UZU ZUU UZC UUZ GUU UZC UG-BHQ1 (2′OMe-RNA)</td>
</tr>
<tr>
<td>Caged Q-Cy3 MB4</td>
<td>BHQ2-PCL-Cy3-CAC GAC UUZ GCU UZC UZU ZUU UZC UUZ GUU UZC UG-BHQ2 (2′OMe-RNA/DNA)</td>
</tr>
<tr>
<td>Caged Q-Atto550 MB5</td>
<td>BHQ2-PCL-Atto550-CAC GAC UUZ GCU UZC UZU ZUU UZC UUZ GUU UZC UG-BHQ2 (2′OMe-RNA)</td>
</tr>
<tr>
<td>Caged Q-TexasRed MB6</td>
<td>BHQ2-PCL-TexasRed-CAC GAC UUZ GCU UZC UZU ZUU UZC UUZ GUU UZC UG-BHQ2 (2′OMe-RNA)</td>
</tr>
<tr>
<td>Caged Q-Atto647N MB7</td>
<td>BBQ-PCL-Atto647N-CAC GAC UUZ GCU UZC UZU ZUU UZC UUZ GUU UZC UG-BHQ2 (2′OMe-RNA)</td>
</tr>
<tr>
<td>Caged Q-Atto647 MB8</td>
<td>BBQ-PCL-Atto647-CAC GAC UUZ GCU UZC UZU ZUU UZC UUZ GUU UZC UG-BHQ2 (2′OMe-RNA)</td>
</tr>
<tr>
<td>Caged Q-Atto647N MB9</td>
<td>BBQ-PCL-Atto647N-CAC GAC UUZ GCU UZC UZU ZUU UZC UUZ GUU UZC UG-BHQ3 (2′OMe-RNA)</td>
</tr>
</tbody>
</table>
### Pre-microRNA Sequences (RNA)

**Table 10: Oligonucleotides for pre-miRNA study.**

- **p** – terminal Phosphate
- **F** – dTFluorophore
- **Q** – dTQuencher
- **O** – tC
- **N** – tC

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native pre-miRNA-181a1</td>
<td>pAAC AUU CAA CGC UGU CGG UGA GUU UGG AAU UCA AAU AAA AAC CAU CGA CCG UUG AUU GUA CC</td>
</tr>
<tr>
<td>Anti-pre-miRNA-181a1 FISH probe</td>
<td>UUU UUA UUU GAA UUC AAA UAA CAU CGA CCG UUG AUU GUA CC</td>
</tr>
<tr>
<td>Ligation part a</td>
<td>pCAA AUA AAA ACC AUC GAC CGU UGA UUG UAC C</td>
</tr>
<tr>
<td>Ligation part b</td>
<td>AAC AUU CAA CGC UGU CGG UGA GUU UGG AAU U</td>
</tr>
<tr>
<td>Splint DNA</td>
<td>GAT GGT TTT TAT TTG AAT TCC AAA CTC ACC (DNA)</td>
</tr>
<tr>
<td>General design b</td>
<td>pAAC AUU CAA CGC UGU CGG UGA GFU UQG GAA UUC AAA UAA AAA CCA UCG ACC GUU GAU UGU ACC</td>
</tr>
<tr>
<td>Fluorescein/Dabcyl probe</td>
<td>pAAC AUU CAA CGC UGU CGG UGA GFU UGG AAU UCA AAU AAQ AAA CCA UCG ACC GUU GAU UGU ACC</td>
</tr>
<tr>
<td>TAMRA/BHQ2 probe</td>
<td>pAAC AUU CAA CGC UGU CGG UGA GFU UGG AAU UCA AAU AAQ AAA CCA UCG ACC GUU GAU UGU ACC</td>
</tr>
<tr>
<td>Pyrene/Perylene probe</td>
<td>pAAC AUU CAA CGC UGU CGG UGA GFU UGG AAU UCA AAU AAQ AAA CCA UCG ACC GUU GAU UGU ACC</td>
</tr>
<tr>
<td>tC/tCnitro probe</td>
<td>pAAC AUU CAA CGC UGU CGG UGA GFU UGU UGG AAU UCA AAU AAN AAA GCA UCG ACC GUU GAU UGU ACC</td>
</tr>
<tr>
<td>Hybrid pre-miRNA-181/150</td>
<td>pAAC AUU CAA CGC UGU CGG UGA GFU UGU UGG CCU CAG AQA AAC CAU CGA CCG UUG AUU GUA CC</td>
</tr>
</tbody>
</table>

Pre-miRNA probes were characterised by PAGE. Alternatively, the mass of sequence derivatives was determined after Dicer-mediated *in vitro* cleavage by ESI-MS of the cleavage products. Proper mass results of the respective full-length products could not be determined reliably.
## HPLC columns

Table 11: List of HPLC columns.

<table>
<thead>
<tr>
<th>Stationary phase material</th>
<th>ID in mm</th>
<th>Length in mm</th>
<th>Manufacturer</th>
</tr>
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<tbody>
<tr>
<td>Nukleosil 100-5 C18</td>
<td>4.6</td>
<td>250</td>
<td>CS chromatography</td>
</tr>
<tr>
<td>Multochrom 100-5 C18</td>
<td>4.6</td>
<td>250</td>
<td>CS chromatography</td>
</tr>
<tr>
<td>Eclipse XDB C18 5 µm</td>
<td>4.0</td>
<td>150</td>
<td>Agilent</td>
</tr>
<tr>
<td>Jupiter 4µ Proteo 90A C12</td>
<td>4.6</td>
<td>250</td>
<td>Phenomenex</td>
</tr>
<tr>
<td>Jupiter 4µ Proteo 90A C12</td>
<td>15</td>
<td>250</td>
<td>Phenomenex</td>
</tr>
<tr>
<td>DNAPac PA200 AEX</td>
<td>22</td>
<td>250</td>
<td>Dionex</td>
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<tr>
<td>DNAPac PA100 AEX</td>
<td>4.0</td>
<td>250</td>
<td>Dionex</td>
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</table>

## Buffers

Table 12: Buffer recipes.

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<thead>
<tr>
<th>Buffer</th>
<th>Concentration</th>
<th>Calculated amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x PBS, pH 7.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>137 mM</td>
<td>8.00 g</td>
</tr>
<tr>
<td>KCl</td>
<td>2.7 mM</td>
<td>0.20 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>100 mM</td>
<td>1.44 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.0 mM</td>
<td>0.24 g</td>
</tr>
<tr>
<td>TEA, pH 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triethylamine (TEA)</td>
<td>1 M</td>
<td>139 mL</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>1 M</td>
<td>57.2 mL</td>
</tr>
<tr>
<td>Phenol saturated TE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris buffer</td>
<td>10 mM</td>
<td>1.21 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>0.29 g</td>
</tr>
<tr>
<td>Reference Number</td>
<td>Author(s)</td>
<td>Journal/Book/Conference Name</td>
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<tr>
<td>(5)</td>
<td>Froehler, B. C.; Ng, P. G.; Matteucci, M. D.</td>
<td>Nucleic Acids Res.</td>
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<td>(13)</td>
<td>Efimov, V. A.; Buryakova, A. A.; Reverdatto, S. V.; Chakhmakhcheva, O. G.; Ovchinnikov, Y. A.</td>
<td>Tetrahedron Lett.</td>
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<td>(23)</td>
<td>Reese, C. B.</td>
<td>Tetrahedron</td>
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<td>(26)</td>
<td>Zhang, X.; Gaffney, B. L.; Jones, R. A.</td>
<td>Nucleic Acids Res.</td>
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</table>
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<td>Schematic view of the base pairing between peptide nucleic acid (PNA) and DNA.</td>
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Danksagung

Prof. Dr. Alexander Heckel danke ich für die Vergabe der interessanten Promotionsthemen und für die Möglichkeit diese Arbeit in seiner Gruppe durchführen zu können.

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Curriculum Vitae

Persönliche Daten

Name:    Jennifer Sarah Rinne
Anschrift:   Am Weigelsgarten 27
            60433 Frankfurt am Main
Geburtsdatum:   12.11.1986
Geburtsort:   Hannover

Praktika, berufliche Erfahrung

10/2011 – heute  Promotion im Exzellenzcluster der Goethe-Universität Frankfurt:
Betreuer: Prof. Dr. Alexander Heckel
„Light-inducible Molecular Beacons and Visualisation of microRNA Maturation in Neurons“

01/2011 – 09/2011  Diplomarbeit: Goethe Universität Frankfurt (Institut für Biochemie):
Betreuer: Prof. Dr. Robert Tampé, Dr. David Parcej
„Functional Purification of the Human Transporter Associated with Antigen Processing and its Core Domain from Pichia pastoris“

12/2009 – 05/2010  Forschungsaufenthalt: Westmead Hospital (Departement for Nuclear Medicine and Radiopharmacy), Sydney, Australien:
Betreuer: Prof. Dr. Vijay Kumar
„68Gallium-labelled Glucosamine as a Potential Compound for the Positron Emission Tomography (PET)“

11/2009  Biochemie-Praktikum: Research School of Biology, Australian National University (ANU), Canberra, Australien:
Betreuerin: Prof. Dr. Ulrike Mathesius

02/2009 – 10/2009  Wissenschaftliche Hilfskraft: Max-Planck Institut für Chemie, Mainz:
Betreuer: Dr. Frank Keppler, Frederik Althoff

Ausbildung

Johannes Gutenberg-Universität, Mainz

09/1999 – 06/2006  St.-Ursula Schule Hannover
Abschluss: Allgemeine Hochschulreife

Stipendien

09/2007 – 09/2011  Friedrich-Naumann-Stiftung für die Freiheit
03/2009 – 09/2011  Studienstiftung des deutschen Volkes
03/2012 – 02/2014  Promotionsstipendium des Fonds der chemischen Industrie
Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt, dass ich die vorgelegte Dissertation selbständig angefertigt und mich nicht anderer Hilfsmittel als der in ihr angegebenen bedient habe, insbesondere, dass alle Entlehnungen aus anderen Schriften mit Angabe der betreffenden Schrift gekennzeichnet sind.

Ich versichere, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

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