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Establishment of a fluorescence *in situ* hybridization-based methodology for the visualization of specific tRNA-derived fragments

Master's Thesis

University of Veterinary Medicine Vienna

submitted by

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Vienna, December 2021

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Plagiarism disclaimer

I declare that this Master's Thesis is my own and autonomous work. All sources and aids used have been indicated as such. All text quoted directly of paraphrased have been indicated by in-text citations. Full bibliographic details are given in the reference list. This work has not been submitted to any other examination authority.

Signed,

Laura Borac 15.12.2021, Vienna, Austria

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Acknowledgements

First of all, I want to thank Matthias for making me a part of his group during the research for my thesis. Besides giving me the opportunity to work on an exciting project, he also motivated me to develop new scientific skills and face new challenges that turned out to be enriching experiences. I learned from him that openness to new ideas and creative solutions are highly beneficial for overcoming technical challenges.

I would also like to thank Aleksej for sharing his contagious enthusiasm about science. He encouraged me to expand my knowledge and think critically. I am grateful for all the valuable input he gave me.

Additionally, I want to thank my former and present colleagues Michaela, Lisa and Nasim, for making our lab (and our office) a great working environment.

Also, many thanks to everybody from the Center for Anatomy and Cell Biology at the Medical University of Vienna. Whenever I had a question or needed help, everybody was friendly and supportive. This institute was an awesome place to start my scientific career and I am very happy for the opportunity to connect with so many interesting people.

Finally, thanks to my family for their love and support throughout all my life. I want to express my greatest gratitude to you for always supporting me and allowing me to find the direction I am really interested in.

List of Abbreviations

ACNases	Anticodon nucleases
ANG	Angiogenin
cDNA	Complementary DNA
FISH	Fluorescence *in situ* Hybridization
HCR	Hybridization Chain Reaction
iAs	Inorganic Arsenite
ISH	*in situ* Hybridization
mRNA	Messenger RNA
nt	Nucleotide
PAGE	Polyacrylamide Gel Electrophoresis
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
tRNA	Transfer RNA
tsRNA	tRNA-derived small RNA

1 Introduction

1.1 Structure, biogenesis and canonical role of transfer RNAs

Transfer RNAs (tRNAs) are the most evolutionarily conserved RNA molecules in all three main domains of life, as well as the most abundant RNAs in cells by number of molecules (Palazzo and Lee 2015). In their canonical role, they are fundamental components of the translational machinery, acting as adaptor molecules that are essential for messenger RNA (mRNA) decoding. tRNAs are generated and processed in highly complex pathways including multiple nucleolytic steps, varying subcellular localizations along with sequence and base modifications.

1.1.1 Structure

tRNA molecules usually consist of 76 to 93 nucleotides with a 5' phosphate group and a 3' hydroxyl group, where a terminal CCA trinucleotide that is added in a non-templated fashion serves as aminoacylation site. Apart from the four RNA bases (adenine, A; cytosine, C; uracil, U; and guanine, G) tRNAs also contain various modified nucleosides (Pan 2018).

In their secondary structure, tRNAs display a distinctive cloverleaf-like shape. The four arms of the cloverleaf are named acceptor stem, dihydrouridine (D)-loop, anticodon (AC)-loop, variable (V)-loop and pseudouridine or TψC (T)-loop. When folded into tertiary structure, tRNA forms an L-shape in which the T-stem stacks on the acceptor stem and the AC-stem onto the D-stem, tertiary interactions between nucleotides within this formation are essential for its stabilization (Giegé 2008).

1.1.2 Biogenesis and Processing

By number of molecules tRNAs are the most abundant RNAs in mammalian cells (Palazzo and Lee 2015). Albeit tRNA gene copy number varies among humans (Iben and Maraia 2014), generally there are around 500 tRNA genes dispersed throughout human genome (Parisien et al. 2013).

In contrast to mRNA biogenesis, tRNA biogenesis starts transcriptionally in the nucleolus, where they are firstly transcribed by the DNA-dependent RNA polymerase III as a primary or precursor tRNA transcript (pre-tRNA). Those pre-tRNAs must undergo several enzymatic processing steps at various subcellular locations to become functional mature tRNAs. The 5' leaders are removed from pre-tRNAs by RNase P, a ribonuclear protein complex, which is known as 5' maturation, while the 3' trailer sequence is removed by the RNase Z enzyme (Kunzmann et al 1998). Directly after 3' end processing, the addition of the CCA trinucleotide takes place on the newly produced 3' end, which is performed by CCA-adding enzymes, which are tRNA nucleotidyltransferases (Deutscher 1982).

Further processing steps take place in the nucleoplasm, the inner nuclear membrane and in the cytoplasm upon tRNA nuclear export, where particular tRNA modifications are added. During the tRNA maturation steps on the mitochondrial surface, introns located in the anticodon loop of particular pre-tRNAs are removed by the splicing endonuclease (SEN) complex, which generates a 5' exon displaying a 2',3' cyclic phosphate and a 3' exon with a 5' hydroxyl group. The two resulting tRNA halves are then ligated by Rlg1/Trl1 in yeast and plants (Phizicky et al. 1986) or by an enzyme complex containing the RtcB-like ligase, HSPC117, in humans (Popow et al. 2011)

1.1.3 Turnover and degradation of tRNAs

tRNAs are believed to be highly stable with a long half-life because of their elaborate structure and complex modifications. However, the values for tRNA half-lives vary greatly depending on the tissue they are expressed in ranging from around 9 hours (Gudipati et al. 2012) up to several days (Phizicky and Hopper 2010). Several tRNA turnover mechanisms have been reported, which mainly contribute to tRNA quality control. These processes can even occur under optimal growth conditions and result in degradation of aberrantly processed, inappropriately modified or mis-folded tRNAs (Phyzicky and Hopper 2010; Parker 2012). In contrast, tRNAs can also be subjected to endonucleolytic cleavage in response to specific stress conditions, resulting in two approximately half-sized tRNA fragments (5' and 3' halves).

1.2 tRNA complexity: more than just translation

Apart from their crucial role in protein synthesis, tRNAs are involved in multiple processes beyond translation. Driven by advances in technology such as RNA sequencing (RNA-Seq) and proteomics, additional tRNA functions and their involvement in various regulatory pathways have been shown, for instance by contributing to cellular stress responses (Lee & Collins 2005) or immune responses (Chiou et al. 2018).

1.2.1 tRNA sequence variants (isoacceptors and isodecoders)

The complexity of tRNAs starts with the variety of tRNA coding genes and the degeneracy of the genetic code. For each of the 20 amino acids there needs to be at least one tRNA. During mRNA decoding, the interactions between the tRNA anticodon and the mRNA codon determine the position of the respective amino acid on a growing polypeptide chain. Since the code is degenerated, there is more than one codon for most amino acids, which resulted in the evolution of tRNA isoacceptors. tRNA isoacceptors are a group of tRNA molecules with different sequences but which will become

aminoacylated with the same amino acid. For example, there are three isoacceptors for tRNA Glycine ($tRNA^{Gly}$); $tRNA^{Gly}$ CCC, $tRNA^{Gly}$ UCC and $tRNA^{Gly}$ GCC. The isoacceptors vary in their sequences and are named after the trinucleotide in the anticodon, while still coding for the same amino acid. Isoacceptors can be further sub-classified as isodecoders, which vary in the tRNA body sequence, while carrying the identical trinucleotide in their anticodon.

1.2.2 tRNA base modifications

tRNA complexity is further expanded by post-transcriptional addition of various chemical modifications. tRNAs are the most heavily modified RNAs with an average of 13 modifications per molecule for nucleus-encoded tRNAs. Mitochondrial tRNAs are less modified with an average of five modifications per molecule. Notably, some tRNAs show an uneven distribution pattern of modifications ranging from three to 17 modifications per specific molecule (Pan 2018). Generally, post-transcriptional modifications on tRNAs are assumed to have a major impact on the function of tRNAs. They contribute to the rigidity and stability of the tRNA (Powell et al. 2015), enhance codon-anticodon interactions on the ribosome thereby improving of decoding accuracy (Guy et al. 2015; Liu et al. 2013) and, importantly, they can affect tRNA identity (Muramatsu et al. 1988; Pütz et al. 1994).

1.2.3 tRNA fragmentation

Recent research showed that tRNA fragments are tRNA-derived sequences that extend tRNA function beyond translation. Endonucleolytic cleavage of tRNAs by specific nucleases is a conserved process observed during stress conditions (Thompson and Parker 2009), which has been associated with the formation of stress-induced non-membranous organelles called stress granules (Emara et al. 2010). Stress conditions such as amino acid starvation (Lee and Collins 2005), oxidative stress (Thompson et al. 2008; Yamasaki et al. 2009), heat shock and gamma-irradiation (Yamasaki et al. 2009), nutritional deficiency (Haussecker et al. 2010), hypoxia and hypothermia (Fu et al. 2009) and many more have been reported to lead to a significant tRNA fragmentation. Endonucleolytic cleavage of mature tRNAs results in tRNA fragments that are no longer engaged with canonical aminoacylation reactions. Cleavage of tRNAs in the anticodon loop is catalyzed by the activity of anticodon nucleases (ACNases) (Thompson and Parker 2009). During the cleavage event, specific endonucleases hydrolyse tRNAs in their anticodon loop resulting in 5’ and 3’ halves. This could lead to exposition of protein binding sites that were previously hidden inside the tertiary structure of the mature tRNA molecule and are now available for novel interactions and thereby would re-purpose tRNA sequences (Schimmel 2017).

1.3 tRNA-derived small RNAs

Recent evidence experimental evidence indicated that tRNA fragments contribute to additional (non-canonical) roles of tRNAs such as mediating cellular responses to various types of stress (Wek et al. 1995). Albeit tRNA cleavage occurs primarily in the anticodon loop it is not limited to this position since cleavage events at other tRNA positions have been observed as well (Kawaji et al. 2008; Lee and Collins 2005; Li et al. 2008; Thompson et al. 2008; Zhang et al. 2009). These events result in shorter pieces of fragmented tRNA (Schimmel 2017) or tRNA-derived fragments (tRFs), which are produced mainly under steady-state conditions (Oberbauer and Schaefer 2018).

One sub-type of stress-induced tRNA fragments is often referred to as translation-interfering tRNAs (tiRNAs) as their function has been linked to translational inhibition and formation of stress granules (Ivanov et al. 2014; Lyons et al. 2016). Despite some inconsistencies in the tRNA fragment nomenclature (Oberbauer and Schaefer 2018), the term tRNA-derived small RNAs (tsRNAs) will be used throughout this thesis for molecules resulting from tRNA cleavage events, since the main focus of this work will be on 5' tRNA halves.

1.4 Biomedical significance of the topic

Research from the last several years has linked stress-induced tsRNAs to multiple biological processes. On a cellular level, tsRNAs were identified to play a role in proliferation (Honda et al. 2015), regulation of stress response (Emara et al. 2010) and cell survival after stress (Ivanov et al. 2014).

There is also evidence that specific stress-induced tsRNAs are connected to viral infections by facilitating viral replication (Deng et al. 2015; Ruggero et al. 2014) and evidence for an activity of tsRNAs as tumor suppressors in breast and colorectal cancer (Goodarzi et al. 2015; Huang et al. 2017).

1.5 Detection of tRNA-derived small RNAs

Emerging evidence indicated that tsRNAs are not only tRNA degradation products but rather play regulatory roles in various physiological and pathological processes (reviewed in Li et al.; Oberbauer and Schaefer 2018). However, current tsRNA detection methods are limited to sequencing and biochemical approaches. Biochemical methods such as northern blotting can be used for detection and separation of tsRNAs from any tissue or cell extract. The abundance of tsRNAs can be assessed by high-throughput sequencing approaches. Nevertheless, these methods required the dissociation of tissues and cells followed by denaturing RNA extraction to make tsRNAs accessible, which leads to a loss of any spatial information as to where tsRNA might function.

1.6 Fluorescence *in situ* hybridization

In situ hybridization (ISH) is a powerful method for labelling and localizing DNA or RNA targets within fixed cells, tissue sections or whole mount samples by using complementary DNA or RNA probes. For quantification and spatial detection of target molecules, these probes can be accessorized with fluorophores hence the technique is termed fluorescence *in situ* hybridization (FISH). There are multiple derivations of the initial ISH method. Depending on the target molecule and on the application, usually a distinction is made between DNA-FISH and RNA-FISH.

The principle behind FISH is based on nucleic acid thermodynamics where two complementary strands of nucleic acids anneal to each other to form a duplex under energetically favourable conditions (Felsenfeld and Miles 1967). The duplex formed by target and probe can have DNA:DNA or RNA:RNA identity or even form a DNA:RNA hybrid (Milman et al. 1967). This facilitates the use of RNA or DNA probes to specifically bind to a target sequence within a biological sample. Detection and visualization of the target can be achieved either directly with probes carrying radioactive (Gall and Pardue 1969) or fluorescent (Rudkin and Stollar 1977) labels or indirectly by using histochemical chromogens (Tanner et al. 2000) or antigen binding (Nagaso et al. 2001). There are multiple ways of utilizing probes for signal creation, the simplest being to use linear probes directly linked to fluorophores but recently also more complex signalling systems have been established (Urbanek et al. 2015).

Since the application of FISH has gained popularity, the possibilities for probe and protocol adaptation have also increased vastly. There are numerous reagents, probe-labelling techniques and designs as well as detection approaches that increase FISH specificity (Huber et al. 2018; Young et al. 2020).

Even though the effectiveness of FISH has been validated in different samples as chemically fixed cells, cryosections and FFPE tissue sections, there are numerous sample types that are still unexplored. In addition to that, the signal yield is strongly dependent on the FISH target.

1.7 RNA-Fluorescence in *situ* hybridization

1.7.1 Conventional RNA-FISH

The development of RNA FISH has broadened the application possibilities of previously developed FISH protocols that have been used mainly for DNA targets. In RNA FISH, the RNA target is hybridized to complementary probes containing fluorophores. The preparation of the samples prior to hybridization is known to be an essential part in any RNA FISH protocol and there is a variety of permutations depending on the purpose. The most commonly modified processing steps are tissue preparation (pre-hybridization), hybridization and washing (post-hybridization) (Young et al. 2020).

All steps need to be adjusted in dependence to sample identity and target, as they are determining the efficiency of signal detection (Young et al. 2020).

1.7.2 Hybridization-Chain-Reaction

Although RNA molecules can be visualized by FISH, the resulting signals are often weak. A method that can be used to amplify signal yield while minimizing false positives is Hybridization-Chain-Reaction (HCR) (Marras et al. 2019). HCR is a nucleic acid-based amplification mechanism that facilitates target recognition and signal amplification without an using enzymes. The approach is based on a chain reaction of hybridization of alternating DNA hairpin molecules that is only triggered by the exposure to a target DNA, which is the initiating sequence (Figure 1). The mechanism underlying the assembly is premised on storage of potential energy in the loops of two hairpin species (Dirks and Pierce 2004).

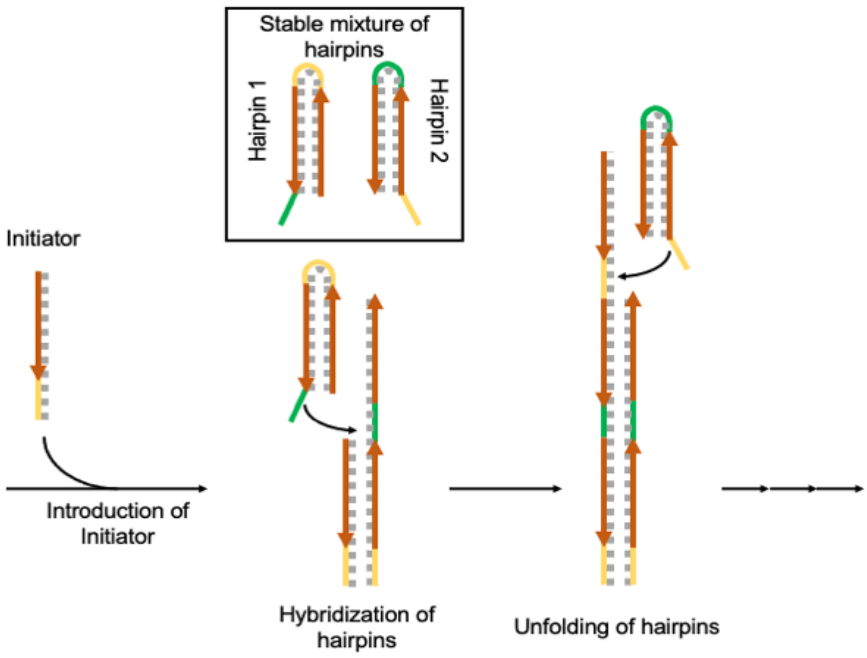


Figure 1. **Schematic of HCR.**

Addition of an initiator sequence to a stable mixture of two hairpin species results in hybridization and unfolding of hairpins. Sequence identity of the hairpins triggers a chain reaction of hybridization events without addition of an enzyme (adapted from Evanko 2004).

2 Aim of the study and Research Question

An unanswered question in the research field of tsRNAs remains where do specific tsRNAs localize in cells, especially during the many processes which tsRNAs have been functionally associated with. This question is still not answered since no technology has been developed that would allow visualizing specific tsRNAs without signal interference from their parental tRNAs.

The main aim of this work was to develop a fluorescence *in situ* hybridization-based methodology that can differentiate between parental tRNAs and specific tsRNAs (here one specific 5' tsRNA) and be used to visualize 5' tsRNAs in cells and different tissues. More specifically this thesis aimed at answering following questions:

1. As sequence identity of tsRNAs is identical to the tRNAs they derive from, is there a possibility of utilizing specific chemical differences between tRNAs and tsRNAs to selectively produce signal from tsRNAs and not tRNAs?

2. And if so, is an approach using synthetic tsRNAs that has been developed *in vitro* and *in vivo* also applicable for detecting stress-induced tsRNAs *in vivo*, for example using stress paradigms or treatment with specific anti-codon nucleases?

3. What signal amplification approaches are efficient and sufficient for tsRNA detection in cell lines?

3 Approaches for visualization of 5’ tsRNAs

Stress-induced endonucleolytic cleavage in the anti-codon loop of tRNAs produces tRNA fragments with the same sequence identity, which is why visualization of tsRNAs by conventional FISH methods fails to distinguish between them. Upon fragmentation, 5’ tsRNAs display a 3’ cyclic phosphate (cycP) moiety upon enzymatic cleavage. This cyclic phosphate is absent in mature tRNAs and therefore a distinctive feature between tRNAs and tsRNAs (Shigematsu et al. 2018).

The 3’ cyclic phosphate of tsRNAs was utilized for sequence-specific ligation of a specific 5’ tsRNA to a complementary linker oligonucleotide (compLINK) using an enzyme, RtcB, which can ligate cycP-containing RNA ends to free hydroxyl groups. This excludes ligation to mature tRNAs and makes compLINKs a suitable platform for signal production *in vivo*.

Another obstacle in visualization of tsRNAs is their low abundance. During stress, only a portion of tRNAs engender specific tsRNAs (Thompson et al. 2008; Yamasaki et al. 2009), making them a rare target for FISH-based approaches. For the development of this approach transfection was utilized to increase the number of tsRNAs in cells artificially, before testing different stress-paradigms. In order to assess transfection efficiency and trace tsRNA signalling *in vivo*, synthetic fluorescently labelled 5’ tsRNAs were liposomally transfected into cells.

For *in vivo* visualization different FISH-based methodologies with compLINKs of varying complexity were applied and their signalling efficiency was compared to determine their applicability for tsRNA detection in cells.

4 Materials and Methods

4.1 Materials

Hazard warning

Some of the following buffers and chemicals can be toxic and/or harmful during handling and must be handled with appropriate protective gear. Handling of toxic components must be performed in a fume hood.

Table 1: Buffers and Solutions

Buffer name	**Composition**
10x Phosphate Buffered Saline (PBS)	1.37 M NaCl 27 mM KCl 100 mM Na_2HPO_4 x $2H_2O$ 18 mM KH_2PO_4
5x Tris-Borate buffer (TB)	446 mM tris base 445 mM ortho-boric acid
20x Saline sodium citrate (SSC)	3 M NaCl 300 mM sodium citrate
Northern-Hybridization buffer	5x SSC 20 mM Na_2HPO_4; pH 7.4 7 % SDS 1x Denhardt's Reagent
Northern-Wash A	3x SSC 5 % SDS
Northern-Wash B	1x SSC 1 % SDS
PK buffer	50 mM Tris-HCl; pH 7.5

	5 mM EDTA
Acetylation solution	0.25% acetic anhydride 0.1M triethanolamine
15% FA hybridization solution	15 % formamide 5x SSC 100 µg/ml heparin 1x Denhardt's solution 0.1 % Tween 20 0.1% CHAPS 5 mM EDTA
30% FA hybridization solution	30 % formamide 5x SSC 9 mM citric acid; pH 6.0 0.1 % Tween 20 50 µg/ml heparin 1x Denhardt's solution 10x dextran sulfate
Hybridization solution w/o FA	5x SSC 9 mM citric acid; pH 6.0 0.1 % Tween 20 50 µg/ml heparin 1x Denhardt's solution 10x dextran sulfate
30% FA probe wash buffer	30 % formamide 5x SSC 9 mM citric acid; pH 6.0

	0.1 % Tween 20 50 µg/ml heparin
HCR hairpin storage buffer	10 mM Tris-HCl; pH 8.0 300 mM NaCl 1 mM EDTA; pH 8.0
HCR amplification buffer	5x SSC 0.1 % Tween 20 10 % dextran sulfate
2x SSCT	2x SSC 0.1 % Tween 20
5x SSCT	5x SSC 0.1 % Tween 20
SSC-washout buffer	50 mM Tris 75 mM KCl 3 mM $MgCl_2$
Citrate buffer	82.5 mM sodium citrate 17.5 mM citric acid
Borate buffer	250 mM tris base; pH 8.8

Table 2: Chemicals

Chemicals	**Supplier**
Acetic Anhydride	Sigma (242845-100G)
Ammonium peroxodisulfate (APS)	Sigma (1.01201.0500)
Ammonium thiocyanate	Sigma (1.012130500)

Bovine serum albumin (BSA)	VWR (1.12018.0100)
Bromophenol blue	VWR (0449-25G)
CHAPS detergent	Roche (10810118001)
Chloroform ($CHCl_3$)	Chemlab (Cl00.0316.2500)
Citric acid	VWR (85514.290)
Dextran sulfate	Sigma (D6001-50G)
Disodium phosphate (Na_2HPO_4)	PanReac AppliChem (A3905.1000)
Dithiotreitol (DTT)	PanReac AppliChem (A11010025)
Ethanol (96 %) (EtOH)	AustrAlco (29.CH00823)
Ethidium bromide (EtBr)	Roth (2218.3)
Ethylene diamine tetraacetate (EDTA)	PanReac AppliChem (A1103.0500)
Ethylene glycol tetraacetate (EGTA)	PanReac AppliChem (A0878.0100)
Ficoll® 400	Sigma (F4375-25G)
Formamide (CH_3NO)	BioChemika (47671)
Glycerol	PanReac AppliChem (151339.1214)
Guanidine thiocyanate	Sigma (G9277-100G)
Heparin	Sigma (H3149-50KU)
Isopropyl alcohol, 2-Propanol	AustrAlco (21.5602.4)
Magnesium Chloride ($MgCl_2$)	PanReac AppliChem (141396.12L)
Methanol (CH_3OH)	VWR (20903.368P)
Milk powder	Maresi (980159)

N- Lauroylsarcosine	Sigma (L9150-100G)
Ortho-Boric acid	VWR (20185.360)
Paraformaldehyde (PFA)	Sigma (P6148-1KG)
Polyacrylamide (19:1) (Rotiphorese® Gel 40)	Roth (3030.2)
Polyacrylamide (37.5:1) (Rotiphorese® Gel 30)	Roth (3029.1)
Polyvinylpyrrolidone (PVP)	PanReac AppliChem (A2259.0250)
Potassium chloride (KCl)	VWR (0395-500G)
Potassium dihydrogen phosphate (KH_2PO_4)	PanReac AppliChem (A1043.1000)
Proteinase K	Neofroxx (2114MG500)
RNase-free H_2O	Thermo Fisher Scientific (10977-035)
Sodium acetate 3-hydrate (NaOAc)	PanReac AppliChem (A1045.1000)
Sodium arsenite ($NaAsO_2$)	Sigma (S7400-100G)
Sodium azide (NaN_3)	Sigma (71290-10G)
Sodium chloride (NaCl)	PanReac AppliChem (A2942.5000)
Sodium deoxycholate	PanReac AppliChem (A1531.0100)
Sodium dodecyl sulfate (SDS)	PanReac AppliChem (A2572.1000)
Tetramethylethylenediamine (TEMED)	Roth (2367.1)
Triethanolamine	Sigma (90279-100ML)
Triton X-100	PanReac AppliChem (A1388.1000)
Tween-20	PanReac AppliChem (72.A4974.1000)
Xylene cyanol FF	Sigma (X4126-10G)

Table 3: Cell culture reagents

Product	**Supplier**
Antibiotic-Antimycotic Solution	Thermo Fisher Scientific (15240062)
DOTAP liposomal transfection reagent	Sigma (11202375001)
DPBS	Thermo Fisher Scientific (14190094)
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific (11960044)
Fetal bovine serum (FBS)	Thermo Fisher Scientific (10500-064)
Geltrex™ basement membrane matrix	Thermo Fisher Scientific (A1413302)
HBSS	Thermo Fisher Scientific (88284)
L-Glutamine	Thermo Fisher Scientific (25030024)
Laminin	Thermo Fisher Scientific (A29248)
Lipofectamine™ 2000 transfection reagent	Thermo Fisher Scientific (11668019)
Opti-MEM™ reduced serum medium	Thermo Fisher Scientific (A4124801)
Viromer® red	Lipocalyx (VR-01LB-00)
Vitronectin	Thermo Fisher Scientific (A14700)

Table 4: Oligonucleotides

Probe name	Sequence (5'-3')	Length (nt)	3' end	5' end	Identity	Application	Purpose
AD_0006	TCT ACC ACT GAA CCA CCA AT	20	-	-	DNA	Northern blot	complementary to 5' tsRNA-Gly^{GCC}
AD_0137	GCA UGG GUG GUU CAG UGG UAG AAU UCU CGC CU	32	cycP	Atto590	RNA	general	5' tsRNA-Gly^{GCC}
LauraB_0002	GCC ATT GAT GGT GCC TAC AG	20	-	-	DNA	RT-PCR	reverse primer
LauraB_0004	rGrGrG rAGG CGA GAA TTC TAC CAC TGA ACC ACC CAT GC	35	Alexa488	rN	RNA/DNA	conventional FISH	complementary to 5' tsRNA-Gly^{GCC}
LauraB_0020	rGrUC GTA TCC AGT GCA GGG TCC GAG GT ATT CGC ACT GGA TAC GAC NNNNNN	50	random hexamer	rN	RNA/DNA	HCR v3.0	Stem loop primer with 3' random hexamer
LauraB_0021	rGrUC GTA TCC AGT GC CTGTAGGCACCATCAAT ACT GGA TAC GAC NNNNNN	49	random hexamer	rN	RNA/DNA	HCR v3.0	stem loop primer with landing site in loop and 3' random hexamer
LauraB_0022	CCT CGT AAA TCC TCA TCA AAA GGC GAG AAT TCT ACC ACT GA	41	-	-	DNA	HCR v3.0	Set 1: Initiator half 1
LauraB_0023	CGA CCC TGC ACT GGA TAC GAC TAA TCA TCC AGT AAA CCG CC	41	-	-	DNA	HCR v3.0	Set 1: Initiator half 2
LauraB_0024	CCT ACA GGC ACT GGA TAC GAC TAA TCA TCC AGT AAA CCG CC	41	-	-	DNA	HCR v3.0	Set 1: Initiator half 2
LauraB_0027	GGC GGT TTA CTG GAT GAT TGA TGA GGA TTT ACG AGG AGC TCA GTC CAT CCT CGT AAA TCC TCA TCA ATC ATC	72	Alexa488	-	DNA	HCR v3.0	Set 2: Initiator half 1
LauraB_0028	CCT CGT AAA TCC TCA TCA ATC ATC CAG TAA ACC GCC GAT GAT TGA TGA GGA TTT ACG AGG ATG GAC TGA GCT	72	-	Alexa488	DNA	HCR v3.0	Set 2: Initiator half 2
LauraB_0029	rGrUC GTA TCC AGT GCC TGT AGG CAC CAT CAA TAC TGG ATA CGA CAG GCG A	49	-	rN	RNA/DNA	HCR v3.0	stem loop primer with landing site in

							loop, specific for 5' tsRNA-Gly^{GCC}
Matt_VIE418	GCA TGG GTG GTT CAG TGG	18	-	-	DNA	RT-PCR	forward primer
Matt_VIE420	CGG GTT GGA GAT ATA GAG TAA TCC CTC TAT ATC TCC	36	Atto488	-	DNA	HCR	Hairpin amplifier 1 Set 1
Matt_VIE421	CTC TAT ATC TCC AAC CCG GGA GAT ATA GAG GGA TTA	36	-	Atto488	DNA	HCR	Hairpin amplifier 2 Set 1
Matt_VIE424	GGC AGG CGA GAA TTC TAC CAC TGA ACC ACC CAT GCA ACT GTA GGC ACC ATC AAT GGC	57	Spacer C3	-	DNA	*in vitro* RtcB ligation	Testing RtcB ligation between RNA and DNA
Matt_VIE425	CTC TAT ATC TCC AAC CCG AA**G CCA TTG ATG GTG CCT ACA G**AA TAA TCC CTC TAT ATC TCC	60	-	-	DNA	HCR	**complementary to HCR landing site**, containing 2 initiator sequences
Matt_VIE426	rGrGC AGG CGA GAA TTC TAC CAC TGA ACC ACC CAT GCA A**CT GTA GGC ACC ATC AAT GGC**	57	Spacer C3	-	RNA/DNA	HCR	complementary to 5' tsRNA-Gly^{GCC} with **landing site** for HCR

Table 5: Other materials

Material	**Supplier**
Microscope slides	Glaswarenfabrik Karl Hecht (10689421)
Microspin-columns with P6-DG	BioRad (7326227)
Microspin S-300 HR columns	Amersham (27513001)
ROTI®Mount FluorCare mounting medium	Roth (HP19.1)
Nylon blotting membrane	GE Healthcare (RPN203B)
SYBR gold	Thermo Fisher Scientific (S11494)
Gelred	BIOTIUM (41008-500)
GoTaq DNA Polymerase	Promega (M300A)
T4 Polynucleotidkinase	Thermo Fisher Scientific (EK0031)
RtcB Ligase	New England Biolabs (M0458S)

4.2 Methods

4.2.1 Cell culture

Maintenance of cell culture

Mouse embryonic fibroblasts (MEF) and cervical cancer (HeLa) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich) supplemented with 1x antibiotic/antimycotics, 10 % FBS (v/v), 2 mM L-Glutamine and maintained in a humidified incubator at 37 °C and 5 % CO_2. For passaging, cells were treated with 0.05 % trypsin-EDTA in DPBS (both Thermo Fisher Scientific).

Growing cells on glass slides

For preparation of glass slides, 18x18 mm glass cover slips (Glaswarenfabrik Karl Hecht) were sterilized and stored in 96 % ethanol. Prior to cell seeding, cover slips were transferred from ethanol into a 6-well plate (1 cover slip per well) and allowed to dry. Afterwards, cover slips were coated with either Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Thermo Fisher Scientific), Vitronectin truncated recombinant human protein (Thermo Fisher Scientific) or recombinant human Laminin 521 (Thermo Fisher Scientific) for two to three hours according to manufacturer's instructions. Subsequently, the 6-well plates were parafilm-sealed and stored at 4 °C until use or used directly. After aspirating the coating solution, MEF or HeLa cells were seeded with a density of about 0.3 x 10^6 cells per well and cultured on cover slips until reaching 70 to 80 % confluency.

Liposomal transfection of RNA with DOTAP

Adherent MEF and HeLa cells were cultured in a culture dish of 35 mm diameter until 70 to 80 % confluency. 2.5 µg or indicated masses of 5' tsRNAs were diluted in a final volume of 25 µl and 15 µl of DOTAP Liposomal transfection reagent (Roche) were diluted in a final volume of 50 µl (both in DMEM). For the transfection mix, the diluted RNA was transferred into the DOTAP solution and resuspended gently. Afterwards, the transfection mix was incubated for 15 minutes at room temperature and inverted every 3 minutes. The culture medium was then replaced with fresh DMEM (1.5 ml for a 35 mm culture dish) containing the transfection mix. After 30 minutes, another 1.5 ml DMEM was added to the cells and incubated for 3 hours, then 2 ml of the medium were removed and replaced by 3 ml fresh DMEM. Incubation at 37 °C over-night followed.

Liposomal transfection of RNA with Lipofectamine

Adherent HeLa cells were cultured in a dish of 35 mm diameter until 70 to 80 % confluency. 1.5 µg or indicated masses of 5' tsRNAs were diluted in Opti-MEM™ reduced serum medium (Thermo Fisher Scientific) and the transfection mix with Lipofectamine™ 3000 Reagent (Thermo Fisher Scientific) was prepared following the manufacturer's instructions. The previous cell culture medium was replaced with 1 ml of Opti-MEM™ medium prior to transfection and the transfection mix consisting of RNA-lipid complexes was added after 20 minutes. Incubation over-night at 37 °C followed.

4.2.2 Induction of tRNA fragmentation

Stress experiments

MEF and HeLa cells were incubated in DMEM supplemented with 0.1 mM, 0.2 mM, 0.3 mM, 0.5 mM and 0.75 mM inorganic sodium arsenite (iAs) for 1 hour. After iAs treatment cells, were washed in Dulbecco's phosphate buffered saline (DPBS; Thermo Fisher Scientific) and incubated in fresh DMEM for another 10 minutes for residual iAs to be released from cells. For stress recovery tests, the medium was then exchanged with fresh DMEM and cells were incubated over-night at 37 °C and on the following day harvested for RNA extraction. For acute stress tests, cells were washed in DPBS after iAs treatment and incubated in fresh DMEM for 10 minutes. For harvesting, medium was aspirated and pre-warmed Trizol (Table 1) was added directly on top of cells, shaken for about 3 minutes and resuspended. The cells in Trizol were collected in Eppendorf tubes and shaken at least for 10 minutes at room temperature.

For all *in situ* hybridization approaches, cells were briefly rinsed in DPBS and treated following the protocol for tissue preparation (Chapter 4.2.6).

Human recombinant Angiogenin

MEF and HeLa were incubated in 1 ml DMEM supplemented with 500 ng of human recombinant angiogenin (hrANG) for 2 or 4 hours. Optionally, cells were recovered from angiogenin treatment by removing the angiogenin-supplemented media and replacing it with fresh DMEM. For RNA extraction after hrANG treatment, cells were briefly washed in DPBS followed by addition of 1 ml of pre-warmed Trizol, shaking for about 3 minutes and resuspension. After collection in Eppendorf tubes, samples were shaken for at least another 10 minutes at room temperature.

For all *in situ* hybridization approaches, hrANG-treated cells were briefly rinsed in DPBS and treated following the protocol for tissue preparation.

4.2.3 RNA methods

RNA extraction from cultured cells

Isolation of total RNA from cultured cells (MEF and HeLa) after induction of tRNA fragmentation was performed by extraction with Trizol. After shaking the samples for at least 10 minutes at room temperature, 200 µl of chloroform were added, shaken vigorously by hand for 30 seconds and centrifuged for 7 minutes at 12.000 g at room temperature. The resulting aqueous phase containing the RNA was re-extracted in equal volumes of chloroform, followed by vigorous shaking by hand for 30 seconds and centrifugation for 5 minutes at 21.000 g at room temperature. The aqueous phase was finally precipitated in one volume of isopropanol supplemented with 1 µl of 15 mg/ml Glycoblue™ at -20 °C (over-night). RNA was pelleted by centrifugation (30 minutes at 21.000 x g at 4 °C, washed once in

75 % ethanol, resuspended in RNase-free water (Thermo Fisher Scientific) and measured using NanoDrop (Thermo Fisher Scientific).

Northern blotting

RNA was separated on a 12 % denaturing urea-polyacrylamide gels, followed by staining with SYBR gold, and RNA transfer onto a Nylon membrane (Roche) using semi-dry blotting in 0.5x TBE (Table 1) for 30 minutes at 10 V=const. Afterwards, blotted RNA was immobilized by UV cross-linking using 120 mJ/cm^2 (UVP Crosslinker CX-2000, Analytik Jena) twice followed by incubation at 60 °C over-night.

Radioactive labelling and Hybridization

The nylon membrane containing immobilized RNAs were pre-hybridized in 10 ml hybridization buffer (Table 1) for at least 1 hour at 39 °C. A T4 PNK forward reaction was used to radioactively label DNA oligonucleotides complementary to tRNA-Gly^{GCC} using p^{32}-γ-ATP (Table 6) by incubating the labelling reaction at 37 °C for 1 hour. T4 PNK was deactivated by a 5-minute incubation at 75 °C. After adding 50 µl of ddH_2O to the reaction, excess p^{32}-γ-ATP was removed from labelled nucleotides by using Bio-Rad Microspin-columns filled with P6-DG desalting gel. Purified probes were added to the hybridization buffer and incubated over-night at 39 °C.

Table 6: Reaction mix for radioactive probe-labelling

Reagent	**Volume [µl]**
DNA Oligonucleotide (10 µM)	0.5
10x T4 Polynucleotide Kinase Buffer A	2
T4 Polynucleotide Kinase (10 U/µl)	1
p^{32}-γ-ATP (10 mCi/ml)	0.5
Water	16
Total	20

Subsequently, membranes were washed with Northern wash A (Table 1) at hybridization temperature for 15 minutes and with Northern wash B (Table 1) at room temperature for 15 minutes. Washed membranes were exposed to storage phosphor screens (GE healthcare) at room temperature and imaged using an Amersham Typhoon Biomolecular Imager (GE healthcare).

4.2.4 Fluorescence *in situ* hybridization and Hybridization Chain Reaction

Oligonucleotide probe (compLINK) designs

In all of the following probe designs, the nucleic acid identity and end moiety of 5' ends played an important role for RtcB ligation. RtcB is an enzyme facilitating ligation between a single stranded RNA with a 3' phosphate or cyclic phosphate (cycP) to another RNA with a 5' hydroxyl group (Tanaka and Shuman 2011), which was exploited in this approach.

For the conventional FISH approach, a 35 nucleotide-long DNA probe (compLINK) complementary to the 5' tsRNA-Gly^{GCC} was designed with a 3' Alexa488-label and four ribonucleotides at its 5' end. The 5' end identity of this fluorescently labelled oligonucleotide was intended to facilitate RtcB ligation between the target tsRNA with a 3' cycP and the hydroxyl group of the 5' ribonucleotide in the RNA/DNA oligo (Figure 2A).

For HCR, a 57 nucleotide-long DNA probe (compLINK) complementary to 5' tsRNA-Gly^{GCC} was designed with two ribonucleotides at the 5'-end. At the 3' end, the DNA probe carried a 20 nucleotide-long overhang with a unique sequence that does not naturally occur in cells. The sequence was adapted from the NEB universal miRNA cloning linker (Lau et al. 2001). This overhang served as a landing site for another probe with complementary to the innermost 20 nucleotides. The two outer 20 nucleotides on both sides surrounding the hybridizing centre-part were designed as HCR initiators. Only in the presence of the HCR initiator sequence, the 36 nucleotide-long hairpin amplifiers are able to assemble in an alternating manner. Fluorescent signal was created through the alternating 3' or 5' ends of the hairpin amplifiers carrying Alexa488 fluorophores (Figure 2B).

For HCR v3.0, different hairpin-compLINKs of 49 to 50 nucleotide length were designed to be a) specific to 5' tsRNA-Gly^{GCC} by making the last 6 nucleotides on the 3' end complementary to the 3' end of 5' tsRNA-Gly^{GCC} or b) non-specific by carrying a random hexamer sequence at the same position. All of the hairpin-compLINKs carried a 5' OH-group for ligation to the 5' tsRNA by RtcB. Upon ligation, the hairpin structure would unfold under denaturing conditions and enable hybridization of split initiators. Different sets of split initiators of either 32 or 41 nucleotide length were designed in such a way that one initiator half would hybridize specifically to the 5' tsRNAs while the other initiator half would hybridize to the (at this stage) unfolded hairpin-compLINK. Upon hybridization of those two initiator halves, the excess sequences not complementary to each other would form the HCR 3.0 initiator platform. Only in cases where initiators correctly hybridized to each other, the platform would provide the exact sequence for initiating HCR amplification by hairpin amplifiers (Figure 2C).

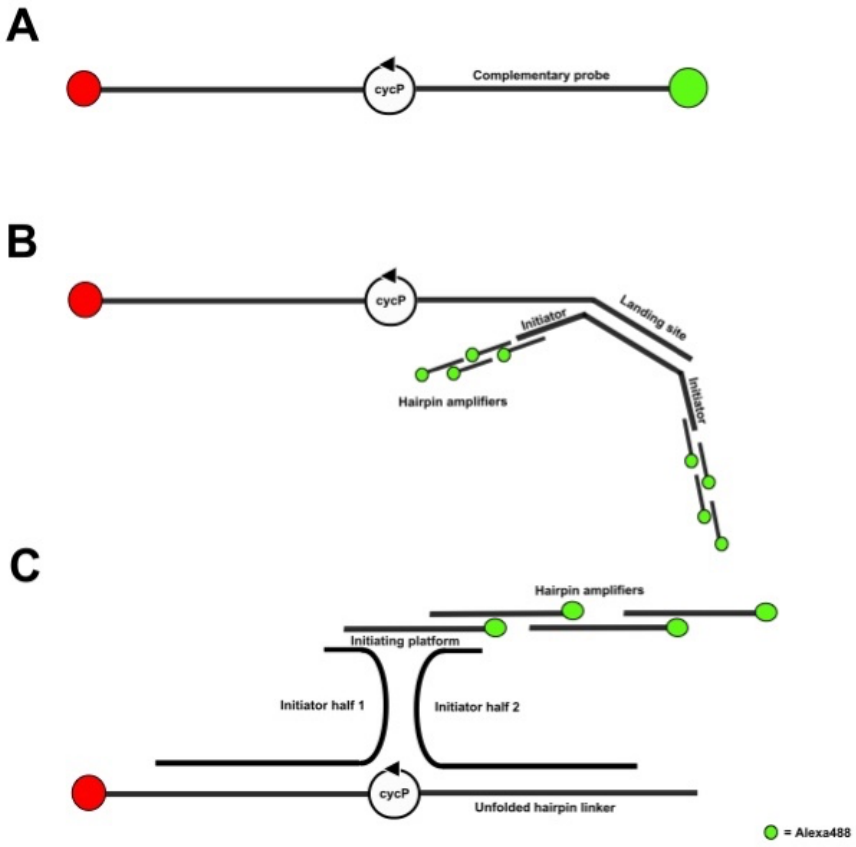


Figure 2. **compLINK designs for different signal amplification methods.**

(A) Conventional fluorescence *in situ* hybridization is based on complementarity of the target to a fluorescent probe. **(B)** HCR is triggered upon hybridization of an initiating probe to the landing site of a compLINK complementary to the 5' tsRNA target. (C) HCR 3.0 requires hybridization of initiator halves to the target and to the unfolded hairpin-compLINK to form an initiating platform where hairpin amplifiers assemble for signal amplification.

4.2.5 In vitro proof of principle testing

Removal of cyclic phosphate by T4 PNK

For validation of the existence of 3' cyclic phosphate moieties (cycP) in synthetic 5' tsRNAs, a treatment with T4 Polynucleotide Kinase (PNK) was performed. Synthetic 5' tsRNA-Gly^{GCC} with cycP and RNA extracted from human embryonic kidney (HEK) cells, that were exposed to 0.5 mM iAs and recovered in normal media for 24 hours, were incubated in following reaction mix (Table 7) for 40 minutes at 37 °C.

Table 7: T4 PNK reaction mix

Reagent	**Volume [µl]**
RNA (1-10 µg/µl)	x
RNasin (40 U/µl)	0.5
10x T4 PNK reaction buffer A	3
T4 PNK	1
Water	up to 30
Total	30

After T4 PNK treatment, phenol-chloroform extraction of RNA was performed by adding 370 µl water and 400 µl acidic phenol (pH 4.5), vigorous shaking and centrifugation at 15.000 g at 4 °C. The upper aqueous phase was transferred into fresh Eppendorf tubes and an equal volume of chloroform was added, followed by shaking and centrifugation as before. Precipitation of RNA was performed as described in section 3.1.2.

Before urea-PAGE, an equal volume of 2x RNA loading dye was added followed by incubation at 75 °C for 3 minutes. RNA was separated on a denaturing urea-PAGE in 0.5x TB and stained with SYBR gold, followed by northern blotting using probes against tRNA-Gly^{GCC}.

RtcB ligation

Ligation by RtcB was tested *in vitro* on synthetic 5' tsRNAs, prior to *in vivo* experimentation.

Synthetic tsRNA-Gly^{GCC} with a 3' cycP was hybridized to compLINKs or hairpin-compLINKs of varying nucleic acid identities. Either they consisted of only DNA nucleotides or were RNA/DNA hybrids in which ribonucleotides were only present as an overhang towards the 5' end of the compLINK. After hybridization, RtcB-mediated ligation between 5' tsRNA and different compLINKs was performed.

For hybridization, 5' tsRNAs and compLINKs were mixed in water, incubated at 95 °C for 3 minutes. RtcB reaction buffer was added, and the mix was ramped down to room temperature using a thermocycler. This step would ensure linearization of the RNA and or DNA and hybridization under ideal conditions. Afterwards $MnCl_2$, GTP and RtcB ligase was added, and the reaction mix was incubated for 1 hour at 37 °C (Table 8).

Table 8: *in vitro* RtcB reaction mix

Reagent	**Volume [µl]**
tsRNA (100 µM**)**	1
compLINK (100 µM)	1
10x RtcB buffer	2
1 mM GTP	2
10 mM $MnCl_2$	2
RtcB ligase	1
Water	11
Total	20

After the ligation reaction, an equal volume of 2x RNA loading dye was added, followed by incubation at 75 °C for 3 minutes. The mobility of ligation products was determined by denaturing urea-PAGE in 0.5x TB followed by staining with SYBR-gold and visualized using a GelDoc device (BioRad).

First ligation tests were performed using commercially available RtcB ligase (NEB). Later ligations were carried out using recombinant RtcB that was expressed by Lisa Koenig and myself followed by purification with the help of Aleksej Drino through a combination of Ni-NTA, QFF anion exchange and Superdex 200_10/300 SEC columns. The concentration of purified RtcB based on molecular absorption coefficients was calculated to be 0.388 mg/ml (6.92 µM). The ligation efficiency of the recombinantly expressed RtcB was assessed in different concentration and compared to commercially available RtcB in ligation reactions using synthetic 5' tsRNA-Gly^{GCC} and a compLINK.

In addition, it was assessed if hairpin-compLINKs, formed by a stem loop primer, could also be ligated to 5' tsRNA, as this would enable the application of HCR 3.0. Hairpin-compLINKs required extra preparational steps prior to RtcB ligation. These included optional dilution in a magnesium-containing buffer and a re-folding step (incubation at 95 °C for 3 minutes). After re-folding, samples were either cooled down at room temperature or ramped down to room temperature using a thermocycler, followed by RNA re-extraction and precipitation as described in section 3.1.2.

Reverse transcription and amplification of RtcB ligation product

Cross-validation of RtcB ligation was carried out by RT-PCR. Ligation products were used directly after incubation with RtcB or extracted from gel. For this step, the ligation products were identified as a SYBR gold positive signal of expected molecular size, excised from a urea-PA gel, shredded, and eluted with 700 µl gel elution buffer at 4 °C over-night on a spinning wheel. The ligation products that were used directly after RtcB ligation was purified with Microspin S-300 HR columns following the manufacturer's instructions. RNA extraction and precipitation was performed as described in section 3.1.2.

Next, first-strand cDNA synthesis was performed. To this end, RtcB ligation products were mixed with a primer, dNTPs and water and denatured for 5 minutes at 65 °C, quickly centrifuged and put on ice promptly. Afterwards, the remaining reagents were added (Table 9) and the reaction was incubated for one hour at 42 °C, followed by inactivation of the RT enzyme for 20 minutes at 65 °C.

Table 9: Reverse transcription reaction mix

Reagent	**Volume [µl]**
Ligation product (5 ng/µl)	1 or 5
Primer (LauraB_0002; 10 µM)	2
10 mM dNTPs	1
Water	up to 10
10x M-MuLV buffer	2
M-MuLV RT (200 U/µl)	1
RNase Inhibitor (40 U/µl)	0.2
Water	6.8
Total	20

Only a fraction of the reverse transcription reaction was used for amplification by PCR. The reverse transcription product was amplified in following reaction mix (Table 10) using a standard PCR program (denaturation at 95 °C for 3 minutes, 35 cycles of template denaturation at 95 °C for 10 seconds, primer annealing at 58 °C for 15 seconds, extension at 72 °C for 15 seconds, final extension at 72 °C for 5 minutes, hold at 15 °C ∞).

Table 10: PCR mix

Reagent	**Volume [µl]**
cDNA	2.5
5x GoTaq green reaction buffer	5
10 mM dNTP	1
10 µM forward primer (Matt_VIE418)	1
10 µM reverse primer (LauraB_0002)	1
GoTaq polymerase (5 U/µl)	0.5
Water	14
Total	25

The PCR product was analysed using 8 % native PAGE followed by SYBR gold staining.

4.2.6 Tissue preparation

After experimental manipulation, cells grown on glass cover slips were prepared for the respective FISH protocols. All steps were performed at room temperature unless indicated otherwise. First, medium was removed from cells, followed by washes in 1x PBS for 10 minutes, fixation with 4 % paraformaldehyde in PBS (PFA) for 10 minutes. After fixation, cells were treated with 0.1 µg/ml Proteinase K in Proteinase K buffer for 10 minutes (Table 1). Cells were washed in 1x PBS for 10 minutes and post-fixed with 4 % PFA for 15 minutes and washed in 1x PBS for 10 minutes again. Subsequently, cells were quickly rinsed in RNase-free H_2O and acetylated twice for 5 minutes with 0.25 % acetic anhydride in 0.1M triethanolamine (acetylation solution). After acetylation, cells were either kept at 4 °C in PBS or citrate buffer until use or immediately used by proceeding to the hybridization step of the FISH protocol.

4.2.7 *in vivo* Hybridization and hybridization environment

Prior to hybridization with the actual probe, pre-hybridization was performed at 37 °C for 30-60 minutes in hybridization solution. Composition of hybridization solution differed depending on the used protocol and application.

For conventional FISH and HCR, all hybridization steps were carried out in 15 % formamide hybridization solution. For HCR 3.0, hybridization with the hairpin-compLINKs was performed in hybridization solution without formamide to prevent unfolding of the hairpin-compLINKs. All other hybridization steps, following RtcB ligation, were performed in 30 % formamide hybridization solution.

Hybridization steps were performed over-night in a humidifying chamber at 37 °C.

4.2.8 *In vivo* post-hybridization treatments

After hybridization over-night, compLINKs and hairpin-compLINKs were removed from cover slips, and cover slips were rinsed in 2x SSCT at 37 °C, washed three times in 2x SSCT at 37 °C for 10 minutes each. Afterwards, cells were washed twice in 5x SSCT at room temperature for 10 minutes each.

When proceeding to RtcB ligation, cover slips were washed twice in SSC-washout buffer (Table 1) at room temperature for 10 minutes each, then incubated in 100 µl pre-ligation buffer mix (Table 11) for 30 minutes at room temperature. For the actual ligation reaction, buffer mix was removed from the cells and cover slips were transferred with cells facing upside-down onto 200 µl droplets of RtcB reaction mix (Table 11) onto a parafilm-covered surface and incubated at 37 °C in a humidifying chamber over-night.

Table 11: *in vivo* pre-ligation and Ligation mix

	Pre-ligation buffer mix	**RtcB reaction mix**
Reagent	Volume [µl]	
10x RtcB reaction buffer	10	20
MnCl2 (10 mM)	10	20
GTP (10 mM)	1	2
RtcB ligase* (6.92 µM)		5
Water	79	153
Total	100	200

* Recombinantly expressed RtcB ligase

For stripping after RtcB ligation, cover slips were first washed three times in 1x PBS for 5 minutes each at room temperature, followed by washing in 2x SSC for 10 minutes at room temperature. For high stringency stripping, cover slips were placed in 70 % formamide + 5x SSC for 30 minutes at 70 °C, then

transferred into pre-chilled (-20 °C) 75 % ethanol for 5 minutes (if not indicated otherwise). Re-hydration of cells was performed by washing three times in 1x PBS for 5 minutes each.

4.2.9 Hybridization-Chain-Reaction

For signal amplification by HCR, cover slips were pre-amplified in HCR amplification buffer (Table 1) for 30 minutes at room temperature. For preparation of fluorescently labelled hairpin amplifiers, both hairpin probes were independently incubated at 95 °C for 90 seconds and cooled down in the dark for about 15 minutes. Then 24 pmol or indicated concentrations of each hairpin amplifier was added to HCR amplification buffer (up to 300 µl per slide). Cover slips were incubated with amplification hairpin mix for 4 hours or indicated times in the dark at room temperature.

Afterwards, excess hairpin amplifiers were rinsed off using 5x SSCT and cover slips were subsequently washed three times in 5x SSCT for 5 minutes each at room temperature. Then, the cover slips were washed three times in 1x PBS for 5 minutes each. Washed cells were incubated with Hoechst 33342 (1 µg/ml) in 1x PBS for 10 minutes at room temperature, followed by washing with 1x PBS for another 10 minutes. Following the washes, cover slips were quickly dipped into distilled water, briefly dried by tapping onto a paper towel and mounted on a microscopic slide with a drop of mounting media (ROTI®Mount FluorCare).

4.2.10 Detection and computational methods

All images were taken by laser scanning microscopy using an Olympus FV3000 confocal microscope and processed using Adobe Photoshop.

5 Results

5.1 *In vitro* testing of the approach

5.1.1 Removal of cyclic phosphate by T4 PNK

To establish an approach based on the 3' cyclic phosphate moiety (cycP) of 5' tsRNAs, synthetic 5' tsRNA-Gly^{GCC} containing a chemically introduced cycP at the 3' end was investigated. Treatment with T4 Polynucleotide kinase led to removal of the cycP in synthetic tsRNAs (Figure 3A), which was detectable as an upshift (mobility change) of the tsRNA signal after urea-PAGE (Figure 3B). These results confirmed that synthetic 5' tsRNAs contained cycP making them suitable substrates for RtcB-mediated ligation reactions.

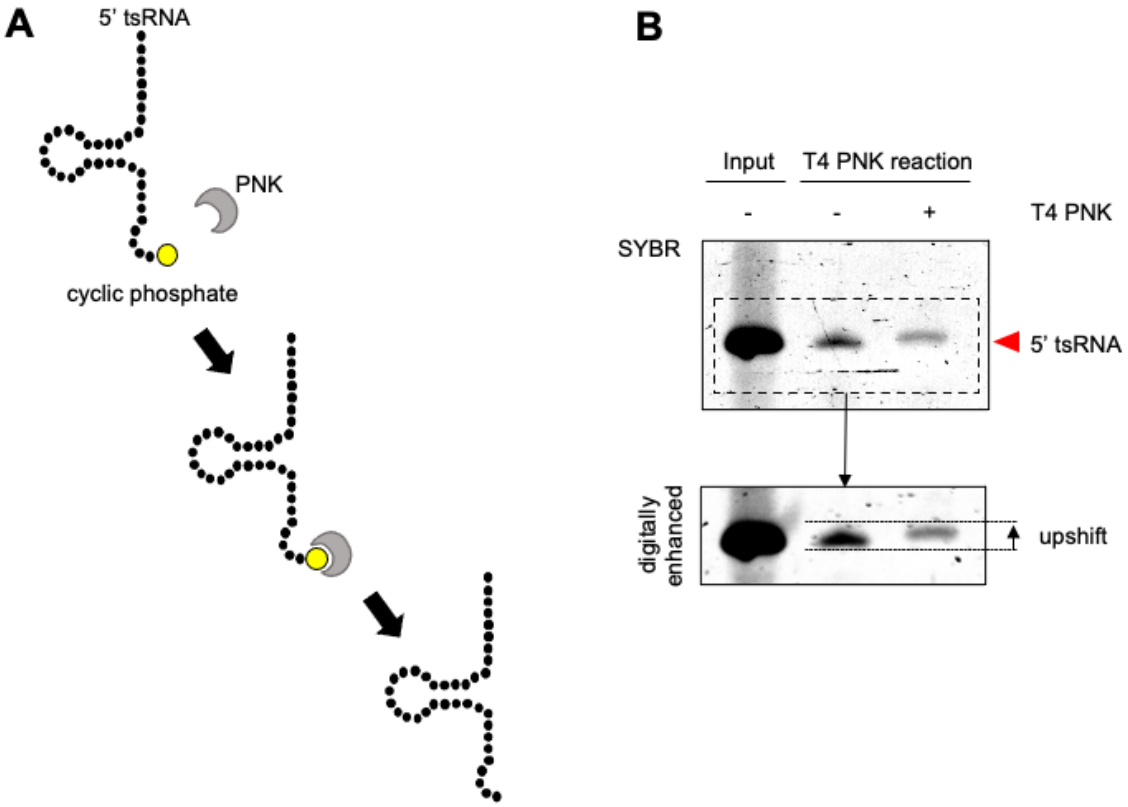


Figure 3. **PNK removes 3' cyclic phosphates from synthetic tsRNAs.**

(A) The 3' phosphatase activity of PNK results in removal of 3' cycP from 5' tsRNAs. **(B)** T4 PNK activity on synthetic 5' tsRNA-Gly^{GCC} was detected as a mobility upshift on a 15% denaturing urea-PAGE followed by SYBR gold staining.

To address if T4 PNK activity can uncover cycP in endogenously produced 5' tsRNAs, RNA was extracted from human embryonic kidney (HEK) cells that were exposed to inorganic sodium arsenite

(iAs), treated with T4 PNK and urea-PAGE. Northern blotting was performed using a complementary probe against the 5' half of tRNA Gly^{GCC} to detect T4 PNK-mediated effects on tRNA fragments (Figure 4A, 4B). The results indicated no clear upshift of stress-induced tsRNAs through T4 PNK treatment suggesting that the existence of cycP cannot be easily monitored by using T4 PNK on cellular tsRNAs.

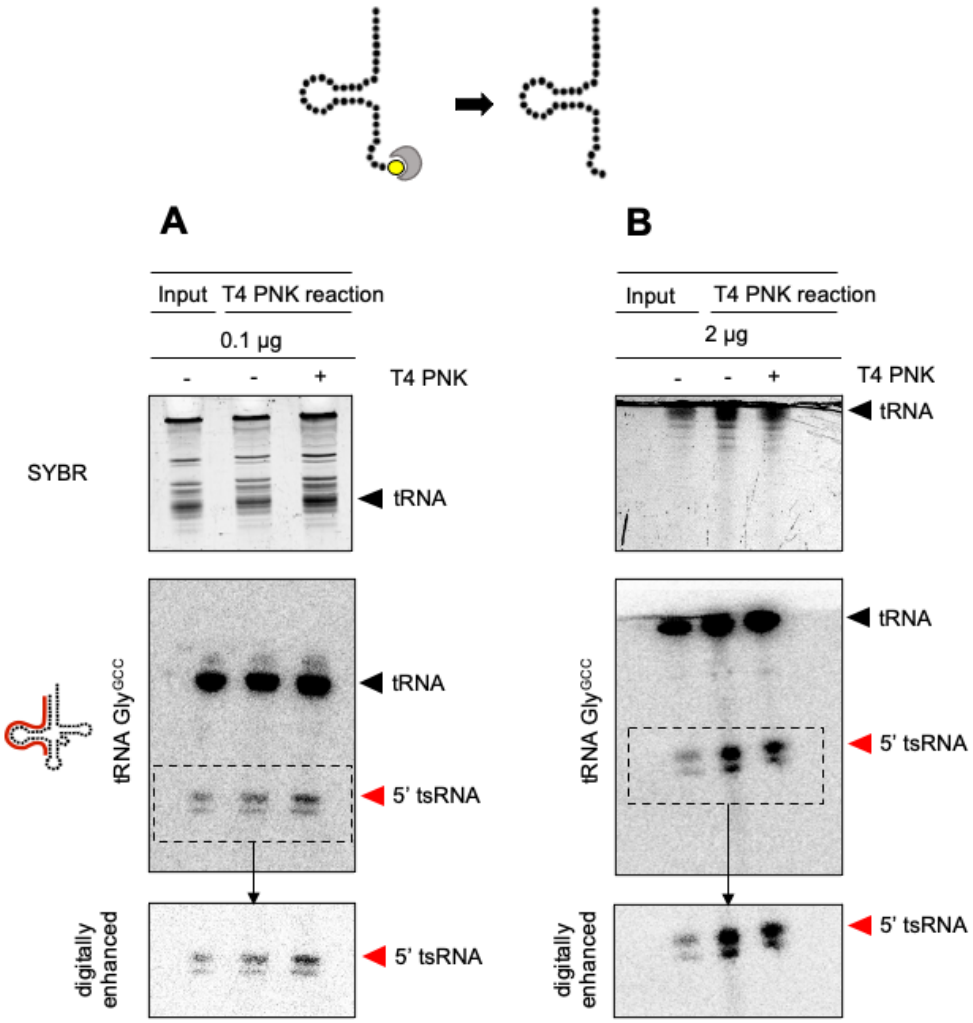


Figure 4. **Test of T4 PNK on endogenously produced tsRNAs.**

RNA was extracted from HEK cells, stressed with 0.5 mM $NaAsO_2$ for one hour and recovered for 24 hours in normal media. T4 PNK activity was assessed on **(A)** 0.1 µg and **(B)** 2 µg of HEK cell-derived RNA, separated on 20% denaturing urea-PAGE with subsequent northern blotting for tRNA-Gly^{GCC}.

5.1.2 RtcB ligation *in vitro*

Prior to *in vivo* experimentation, ligation properties and reaction conditions for RtcB ligase were tested *in vitro* using synthetic tsRNAs and a linker oligonucleotide. RtcB covalently joins single-stranded RNAs with a 3’ terminal cyclic phosphate (cycP) to another RNA with a 5’ terminal hydroxyl group (OH) which was the basis of the approach (Figure 5).

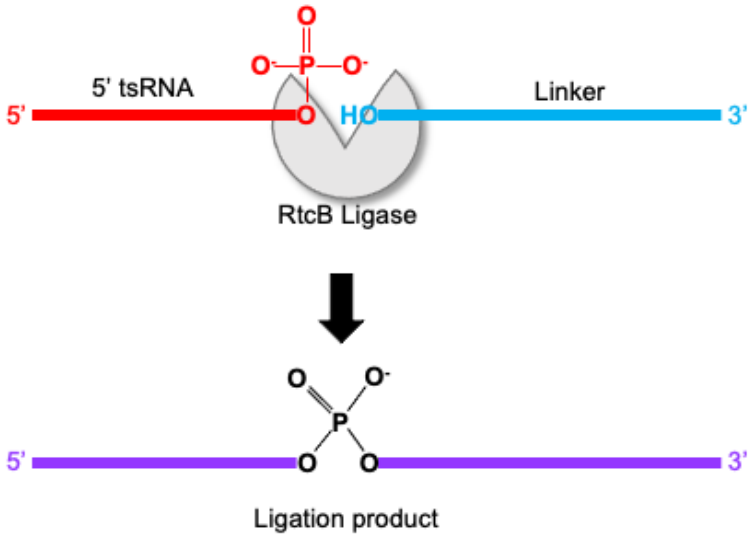


Figure 5. **RtcB ligates 5’ tsRNAs to a complementary linker oligonucleotide.**

The cycP moiety at 5’ tsRNA-Gly^{GCC} was utilized for ligation to a complementary synthetic RNA/DNA hybrid linker oligonucleotide (compLINK) by RtcB ligase. Ligase activity between synthetic tsRNAs and compLINK was assessed *in vitro* by urea-PAGE, followed by SYBR staining. Analysis of a PAGE allowed assessment of ligation efficiency by an upshift of the ligation product due to changed mobility during the electrophoresis. The ligation product comprised of the 5’ tsRNA and the compLINK was therefore detectable by the additive length of two. In addition, compLINK concentration was increased to test if ligation efficiency could be optimized (Figure 6).

The results showed that RtcB ligation of both components was successful and could be visualized using urea-PAGE. SYBR staining confirmed ligated product only in the presence of RtcB ligase. The ligation product could be identified by its length and by a mobility upshift caused by higher molecular weight of the ligation product. Increasing concentration of compLINK by a factor of three did not lead to improved ligation efficiency by RtcB.

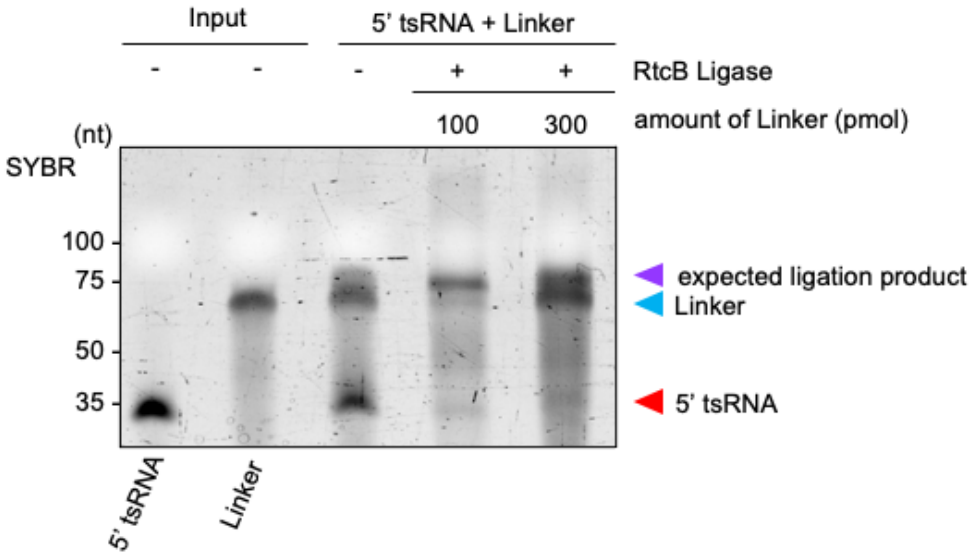
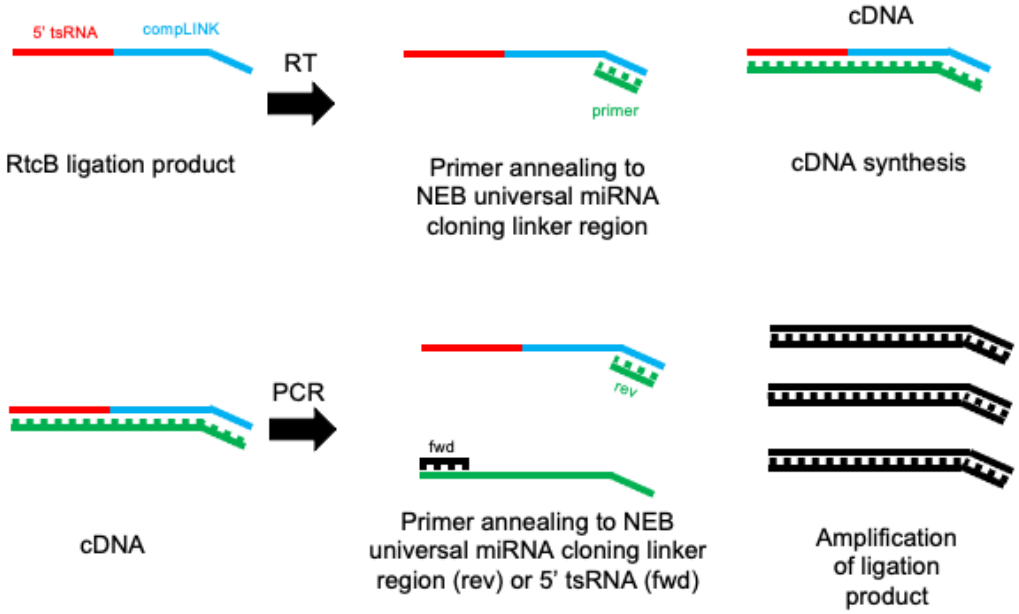


Figure 6. **Test of RtcB ligation *in vitro*.**

100 pmol of synthetic 5' tsRNA (32 nt) was hybridized to 100 pmol and 300 pmol of a complementary linker oligonucleotide (57 nt) and afterwards ligated by RtcB ligase. The ligation efficiency was assessed on a 10% denaturing urea-PAGE with SYBR gold staining. Successful ligation of both components led to a ligation product (89 nt) of higher molecular weight, visible as an upshift of bands.

To cross-validate the identity of the ligation product from RtcB ligation, RT-PCR was performed on a fraction of the ligation product using a reverse transcription primer located at the end of the compLINK (Figure 7). Analysis of SYBR staining showed multiple PCR products with one major product at the length of the expected ligation product (89 bp). These results confirmed that RtcB had covalently linked a synthetic 5' tsRNA containing a CycP with a linker oligonucleotide.



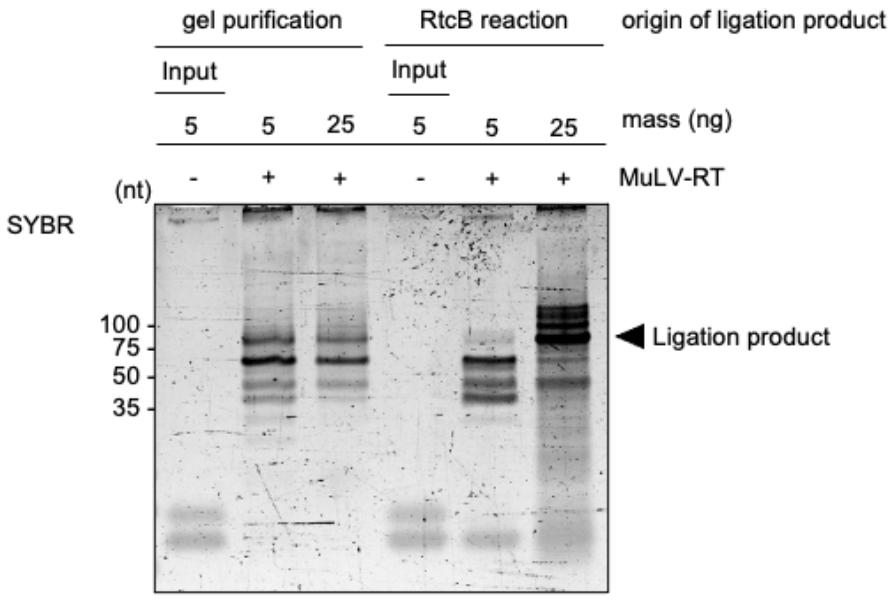


Figure 7. **Validation of *in vitro* ligation by RT-PCR.**

RtcB ligation product from 5' tsRNA and linker was extracted from a 15% denaturing urea-PAGE or used directly upon RtcB reaction for first strand cDNA synthesis by M-MuLV RT followed by PCR amplification. The PCR product was tested on an 8% non-denaturing polyacrylamide gel and SYBR gold stained.

For further designing of the identity of useful linker oligonucleotides and to gain more insight into RtcB activity, it was important to test the selectivity of the used ligase. RtcB is advertised as a ligase linking RNAs exclusively to RNAs. This aspect was incorporated in the compLINK design. To test if RtcB ligation only occurs between two RNAs, ligation between synthetic 5' tsRNAs and compLINKs with different nucleic acid end identities was performed and analyzed by urea-PAGE (Figure 8).

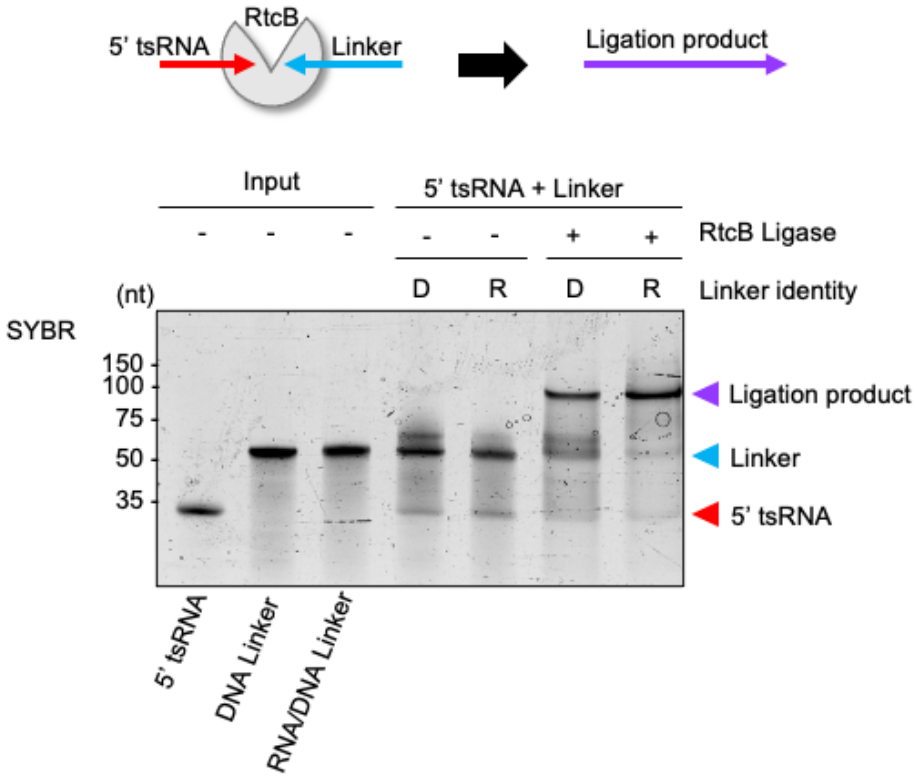


Figure 8. **RtcB ligation between 5' tsRNAs and compLINKs of different nucleic acid end identity.**

10 pmol of synthetic 5' tsRNA (32 nt) was hybridized and ligated to 10 pmol of a complementary linker with full length DNA identity (D; 57 nt) and to 10 pmol of an RNA/DNA linker hybrid with two RNA nucleosides at the 5' end (R; 57 nt). The ligation efficiency was assessed on a 20% denaturing urea-PAGE with SYBR gold staining.

Surprisingly, the results showed a ligation product of expected length (89 nucleotides) for both, DNA and RNA/DNA hybrid, compLINK designs, which indicated that RtcB activity is not limited to ligation between ssRNA and other RNAs, but also occurs between ssRNA and ssDNAs.

With regards to the planned use of this approach *in vivo*, the optimal concentration of compLINKs was tested and once again analyzed on a denaturing urea-PAGE. Specifically, it was determined which ratio

of 5’ tsRNA to compLINK resulted in the highest ligation efficiency. To this end, different amounts of compLINK were ligated to a constant concentration of 5’ tsRNAs. This parameter was tested for the previously described RNA/DNA linker oligonucleotide, which would be used later in HCR signal amplification *in vivo* (Figure 9A) as well as for a compLINK with a 3’ Alexa488-label, which was designed as a fluorescent probe for “classic” fluorescence *in situ* hybridizations (Figure 9B).

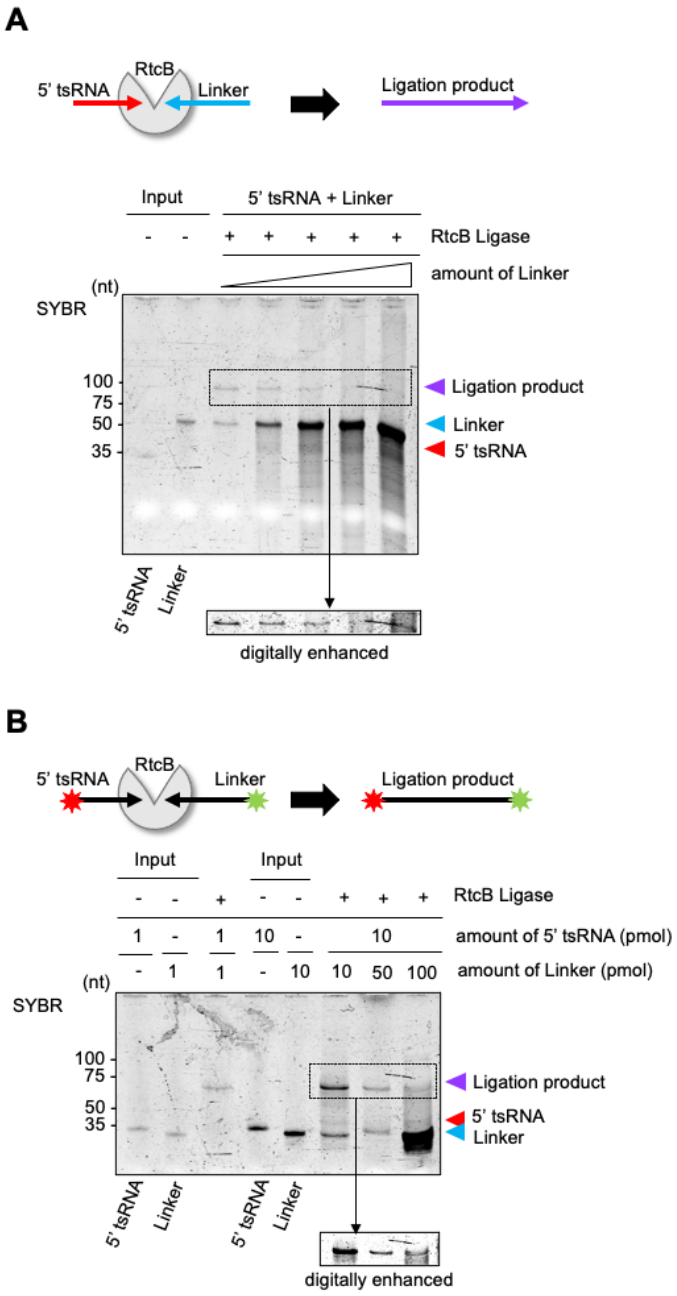


Figure 9. **Linker concentration affects ligation efficiency.**

(A) 1 pmol of synthetic 5' tsRNA (32 nt) was hybridized and ligated to 1, 10, 50, 100 and 200 pmol of a complementary linker oligonucleotide (57 nt). The ligation efficiency was assessed on a 20% denaturing urea-PAGE with SYBR gold staining. The area surrounded by a dashed window was digitally enhanced in the box below. **(B)** Indicated amounts of a Atto590-labeled synthetic 5' tsRNA (32 nt) were hybridized and ligated to indicated amounts of an Alexa488-labeled complementary linker oligonucleotide (35 nt). The ligation efficiency was assessed on a 20% denaturing urea-PAGE with SYBR gold staining. The area surrounded by a dashed window was digitally enhanced in the box below.

SYBR staining indicated that the ligation products decreased in intensity with increasing compLINK concentration and constant 5' tsRNA concentration. Un-ligated components were visible at the same molecular weight as the input. These results showed that compLINK concentrations exceeding 5' tsRNA concentrations by a factor of ten resulted in a significantly lower ligation efficiency, which needed to be considered when using compLINKs *in vivo*.

For economic reasons, instead of buying RtcB from commercial vendors, recombinant RtcB was purified in house and its ligation efficiency was compared to commercial RtcB in ligation reactions of synthetic 5' tsRNA and compLINK (Figure 10). SYBR staining showed that recombinantly expressed RtcB showed comparable ligation efficiency to RtcB purchased from NEB.

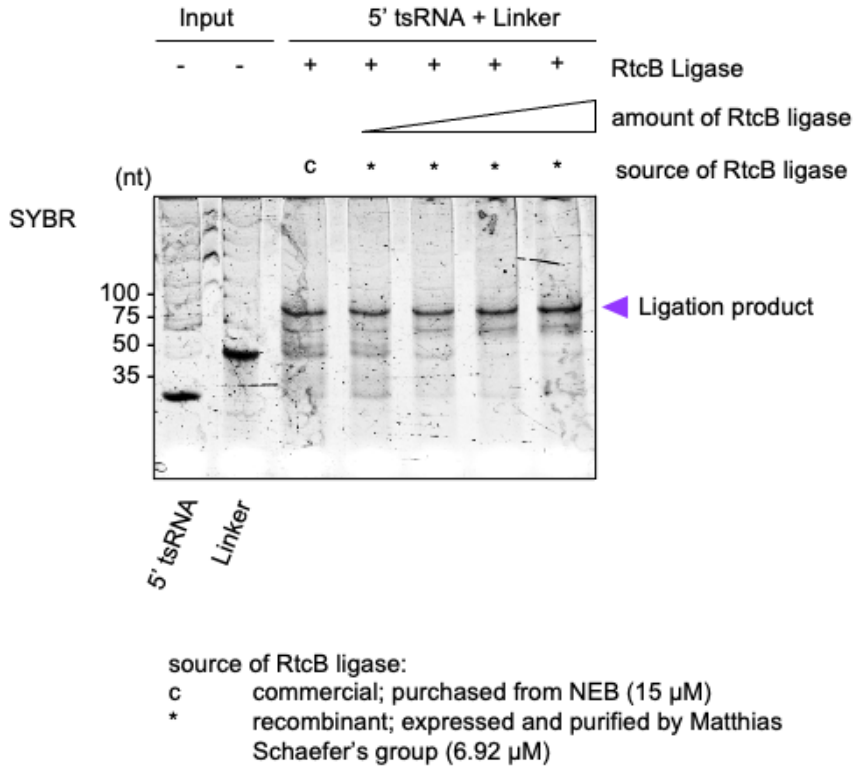


Figure 10. **Comparison of commercial RtcB and recombinantly expressed RtcB.**

Recombinantly produced RtcB protein activity was assessed and compared to commercial RtcB ligase activity by hybridizing and ligating synthetic 5' tsRNA (32 nt) to a linker oligonucleotide (57 nt). For validation of the calculated concentration of the recombinantly expressed RtcB, ligation efficiency of 1, 2, 3, and 4 µl were tested next to 1 µl of commercially purchased RtcB. Ligation efficiency was tested on a 20% denaturing urea-PAGE and stained with SYBR gold.

After assessing RtcB activity on compLINK designs for conventional FISH and HCR, ligation efficiency of hairpin compLINKs for the HCR 3.0 approach to synthetic 5' tsRNAs were tested. As the hairpin-compLINK designs differed from the previous compLINK in terms of complexity (Figure 11), more preparational steps were required prior to ligation.

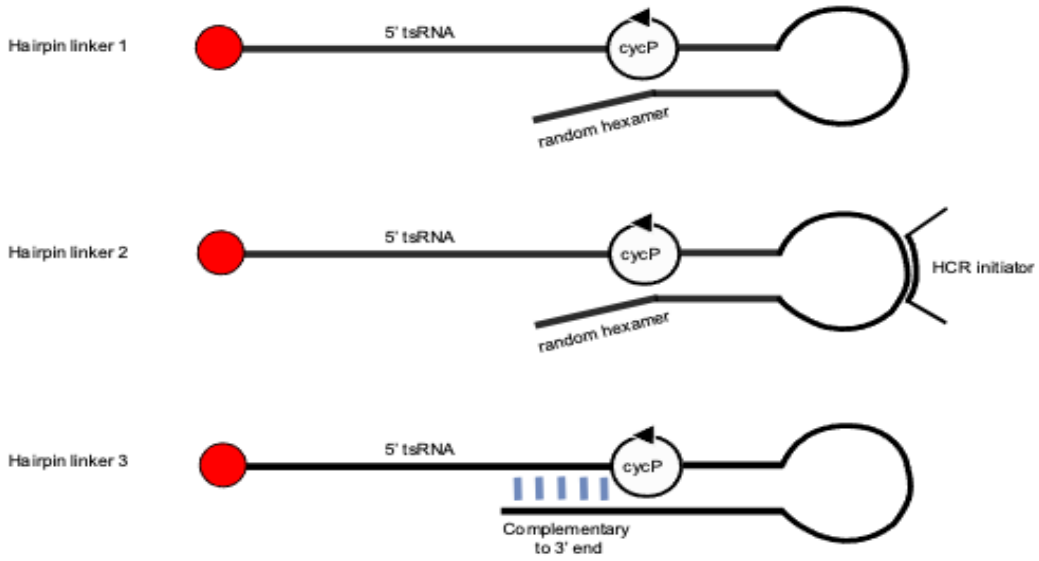


Figure 11. **Hairpin-compLINK designs.**

Correct re-folding of the hairpins had to be ensured prior to ligation, since the formation of a hairpin compLINK was considered to be crucial for ligation efficiency. Different approaches were tested to achieve re-folding of hairpin-compLINKs. However, most of those tests revealed that hairpin-compLINKs could barely be visualized by SYBR staining (Figure 12).

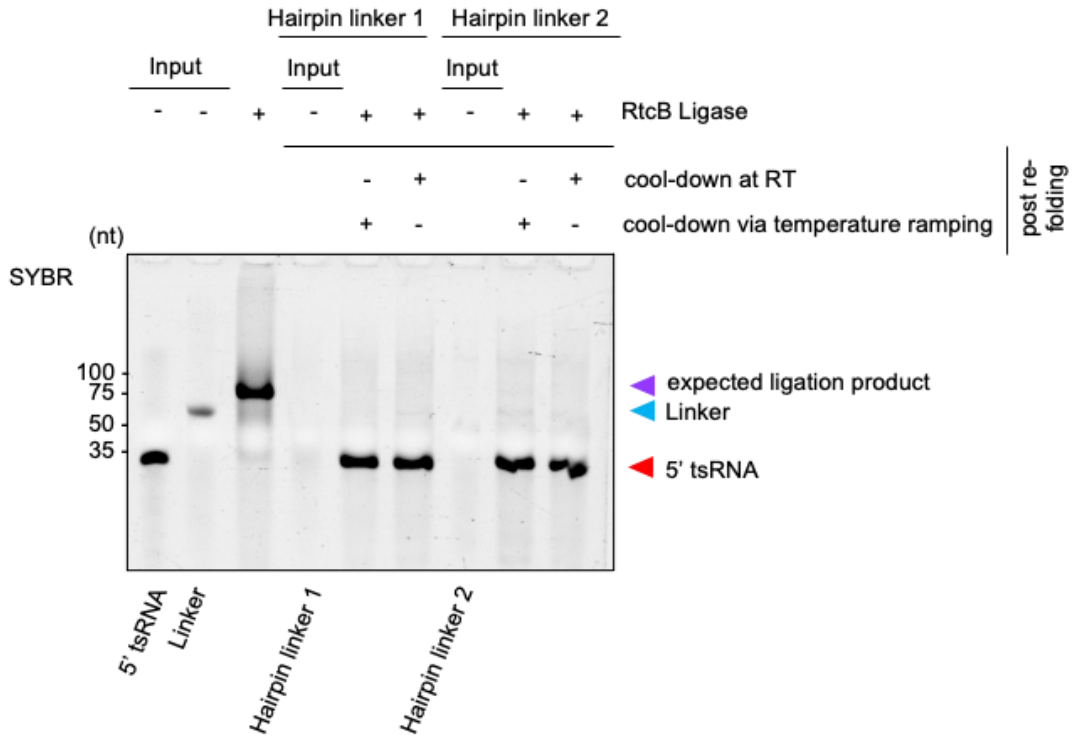


Figure 12. **Difficulties with hairpin oligonucleotide visualization *in vitro*.**

100 pmol synthetic 5' tsRNA (32 nt) were hybridized and ligated to 100 pmol of two different hairpin-compLINK designs (Hairpin linker 1 (50 nt) and Hairpin linker (49 nt) and to 100 pmol of previously used linker (57 nt) as a reference. Hairpin-compLINKs were prepared by including a re-folding step (incubation at 95 °C for 3 minutes) prior to addition of synthetic 5' tsRNA. After re-folding samples were either cooled down at room temperature or ramped down to room temperature using a thermocycler. The ligation efficiency was assessed on a 12% denaturing urea-PAGE with SYBR gold staining.

Specifically, SYBR staining did not enable visualization of the hairpin-compLINK input or the expected ligation products. However, comparing the signal intensity of 5' tsRNA input and the remnant of 5' tsRNAs in RtcB ligation reactions suggested that no ligation reaction to hairpin-compLINKs had occurred.

To address the difficulties with visualizing hairpin-compLINKs after urea-PAGE, the preparational steps were adjusted and ligation was repeated. Adjustment of preparational steps included RNA extraction and re-precipitation from different volumes (Figure 13A) of a magnesium-containing buffer as well as comparisons between precipitation from water versus a magnesium-containing buffer (Figure 13B) for different hairpin-compLINKs.

In the first case, SYBR staining did not enable clear identification of the ligation products, likely due to the formation of a shadow-like haze above the expected length of the ligation products. In addition, formation of polymers of higher molecular weight could be observed in both linker designs, which were likely a product of self-assembly of hairpin-compLINKs.

In the second experiment, SYBR staining enabled identification of hairpin-compLINK 3 as weak signal at the expected length in the input lanes for both precipitation techniques. Furthermore, ligation between 5' tsRNA and hairpin-compLINK 3 could be identified (expected length of 81 nt) only when precipitation of re-folded hairpin-compLINK 3 was performed in $MgCl_2$-containing buffer.

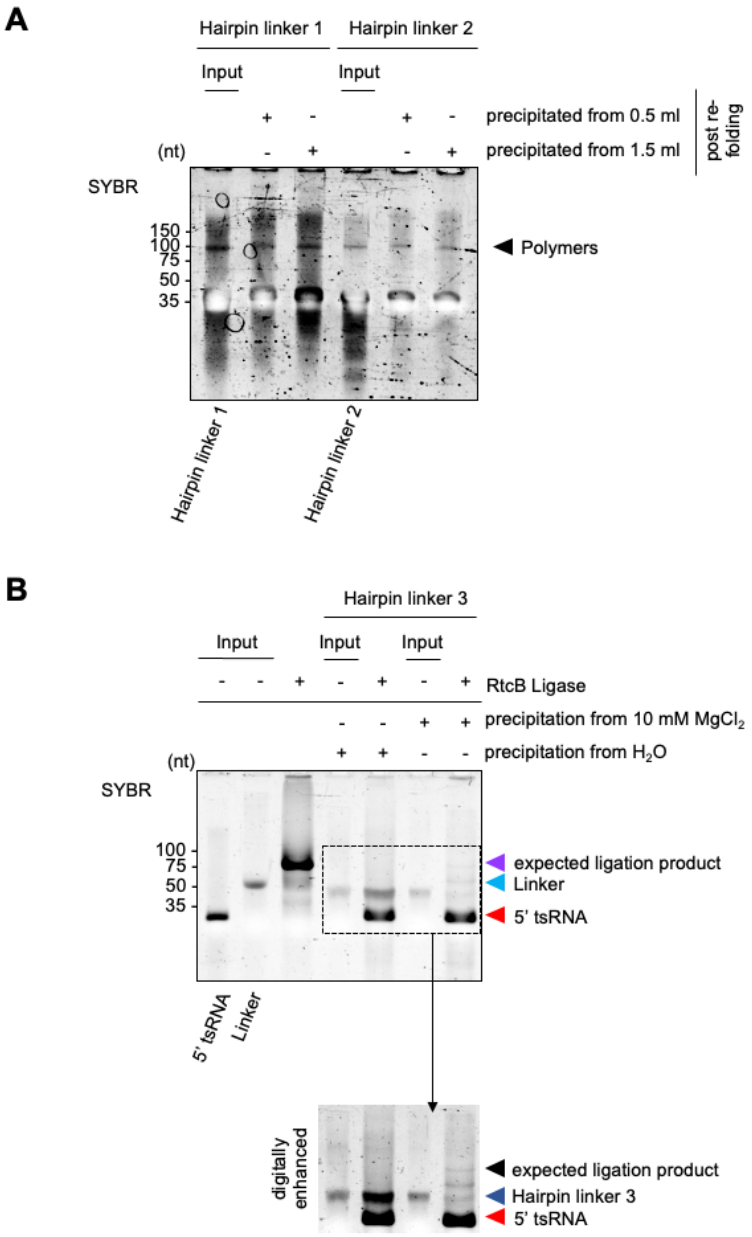


Figure 13. **Adjustment of preparational step enables visualization of hairpin-compLINKs.**

(A) 3 µg of each hairpin-compLINK were diluted in 0.5 ml or 1.5 ml 10x RtcB reaction buffer, re-folded at 95 °C for 3 minutes and cooled down at room temperature. Afterwards they were precipitated in 1 volume isopropanol, 1/10 volume 3M NaOAc and 1 µl glycogen. Pellets were taken up in 10 mM $MgCl_2$. Hairpin-compLINK mobility was assessed on a 12% denaturing urea-PAGE with SYBR gold staining. **(B)** 100 pmol synthetic 5' tsRNA (32 nt) were hybridized and ligated to 100 pmol of a hairpin-compLINK designed specifically for 5' tsRNA-Gly (hairpin-compLINK3 (49 nt) and to 100 pmol of previously used linker (57 nt) as a reference. The hairpin-compLINK was prepared by adding 100 pmol of linker to either 10 mM $MgCl_2$ or water, re-folded at 95 °C for 3 minutes and cooled down at room temperature, whereas $MgCl_2$ (final concentration 10 mM) was added to the samples precipitated from water only for the cool-down phase. Afterwards they were precipitated in 1 volume isopropanol, 1/10 volume 3M NaOAc and 1 µl glycogen. Pellets were taken up in water. Hairpin-compLINK mobility and ligation efficiency between hairpin-compLINK and 5' tsRNAs was assessed on a 15% denaturing urea-PAGE with SYBR gold staining.

To test if the difficulties with hairpin-compLINK identification resulted from the use of the specific SYBR dye, a dilution titration of all three hairpin-compLINK designs was tested on urea-PAGE and subsequently stained with SYBR gold or Gelred (Figure 14A).

The comparison showed that the shadow-like haze above the expected length of hairpin-compLINK1 and 2 (as in Figure 13A) repeatedly occurred in SYBR staining, whereas staining with Gelred did not result in such and allowed visualization of the hairpin-compLINKs in gels. Formation of polymers of higher molecular weight were observed in both staining techniques, yet with SYBR staining they showed higher intensity.

In vitro ligation was subsequently repeated with all 3 hairpin-compLINK designs and analysis by urea-PAGE showed no ligation efficiency with the exception of minor ligation products when using hairpin-compLINK3, specific for 5' tsRNA-Gly^{GCC}, which was visible as a faint ligation product at 81 nt (Figure 14B). Un-ligated 5' tsRNAs were visible as signals of the same molecular size as the input. There were no indications that ligation was affected by increasing hairpin-compLINK concentration.

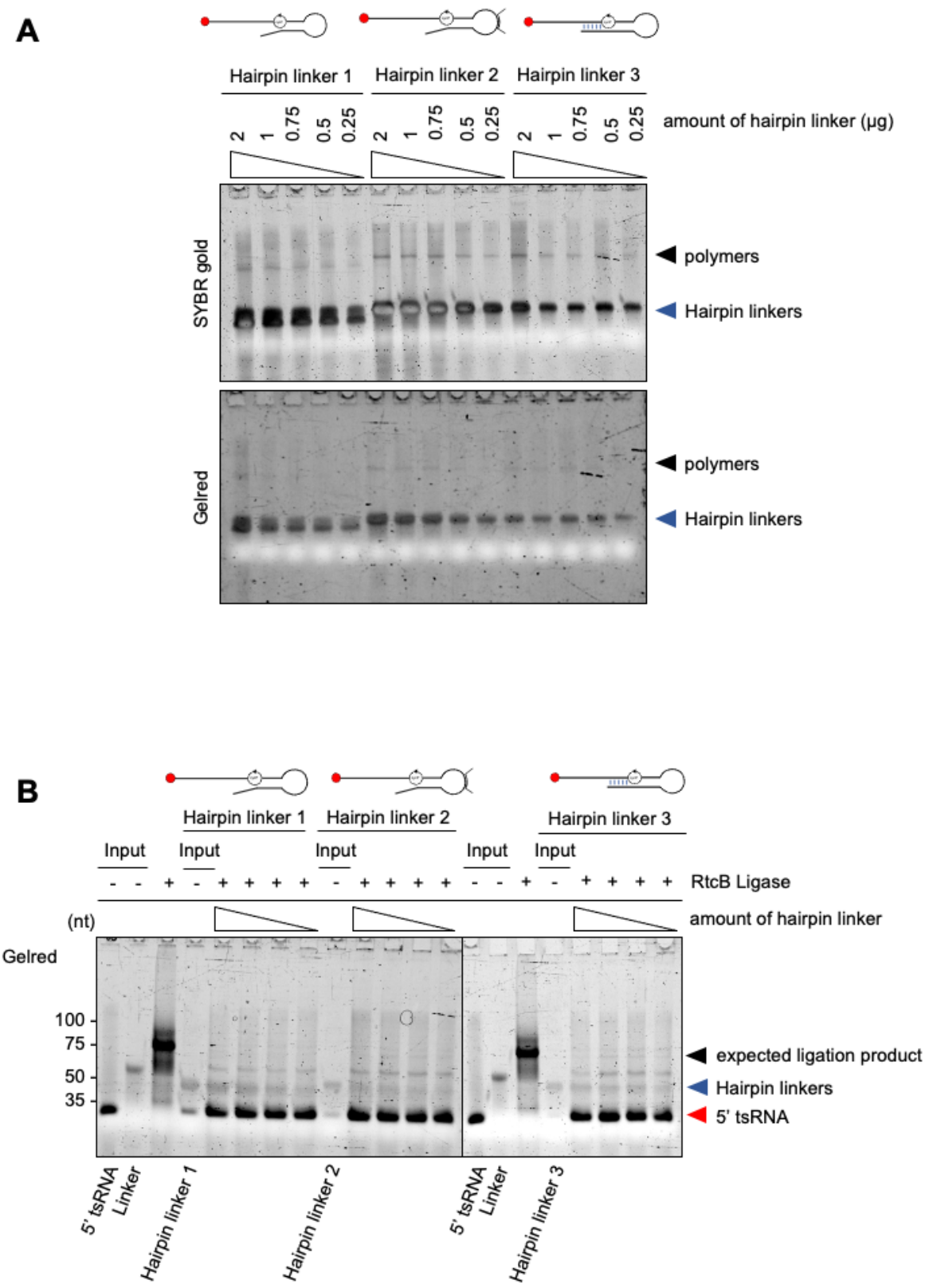


Figure 14. **Gelred staining is more suitable for identification of hairpin-compLINKs.**

(A) Indicated amounts of all three hairpin-compLINK designs were separated on 15% denaturing urea-PAGE and stained with SYBR gold and Gelred. **(B)** 50 pmol (0.55 µg) of synthetic 5' tsRNA (32 nt) was hybridized and ligated to 0.25, 0.5, 0.75 and 1 µg of different hairpin-compLINKs (Hairpin linker 1: 50 nt, Hairpin linker 2: 49 nt, Hairpin linker 3: 49 nt) and to 50 pmol (0.88 µg) of previously used linker (57 nt) as a reference. The ligation efficiency was assessed on a 15% denaturing urea-PAGE with Gelred staining.

5.2 *In vivo* tests using murine cells

5.2.1 Assessment of transfection efficiency

For establishing single parameters of the FISH-based protocol for visualization of specific 5' tsRNAs, multiple aspects were tested and adapted. Due to the low abundance of endogenously produced tsRNAs, the protocol was first optimized after transfecting synthetic 5' tsRNA-Gly^{GCC} containing a 5' Atto590-label and a 3' CycP into various cell lines.

To this end, transfection efficiency was first tested in mouse embryonic fibroblast (MEF) cells. The main aim of the experiment was to determine optimal transfection duration, concentration of the transfected 5' tsRNAs and localization of the transfected tsRNAs (Figure 15).

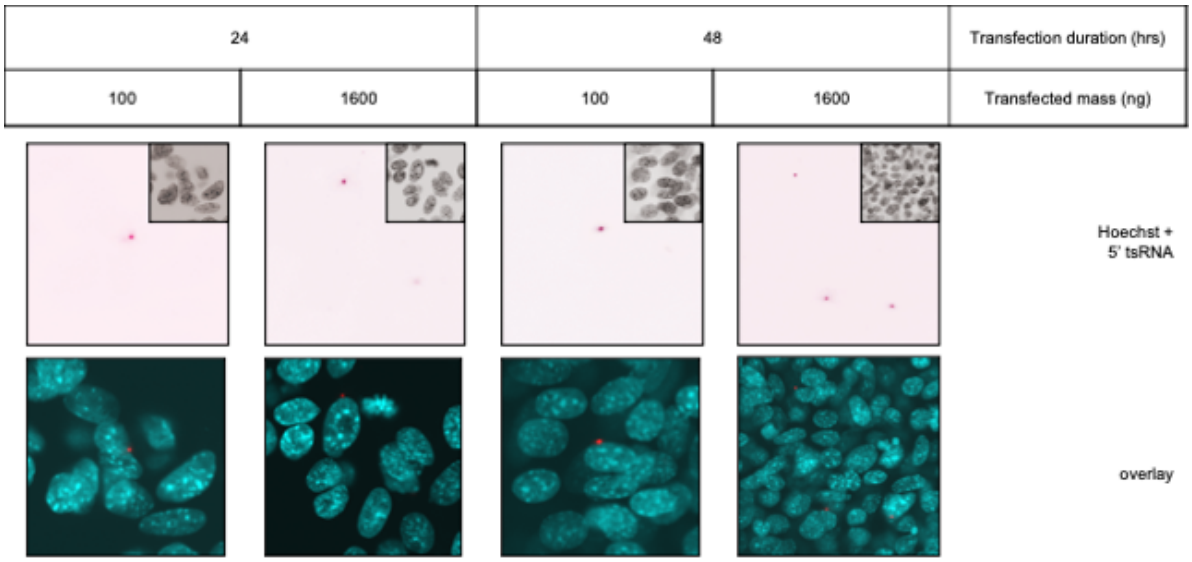


Figure 15. **Fluorescently labelled 5' tsRNAs are detectable post-transfection *in vivo.***

Indicated amounts of Atto590-labeled synthetic 5' tsRNA-Gly^{GCC} were liposomally transfected into mouse embryonic fibroblast (MEF) cells using DOTAP transfection reagent. The cells were kept in transfection media for 24 or 48 hours at 37 °C, afterwards fixed with 4 % PFA and stained for nuclei by Hoechst. Analysis for localization of the transfected product by confocal microscopy followed.

The results showed that transfected 5' tsRNA-Gly^{GCC} appeared as dot-like formations *in vivo*. Localization of dots was observed mainly around the nucleus at the cytosolic phase. Increasing amounts of transfected 5' tsRNA-Gly^{GCC} resulted in increased dot-like formations, whereas doubling the transfection duration did not change the signal collected from transfected 5' tsRNAs.

5.2.2 Comparison of conventional FISH with Hybridization-Chain-Reaction

To assess if conventional FISH methods can be used to detect transfected 5' tsRNAs, MEF cells were subjected to conventional FISH using different amounts of a fluorescent DNA probe (Figure 16). This approach aimed towards testing co-localization of the Atto590-labled 5' tsRNA-Gly^{GCC} and the Alexa488-labeled complementary DNA probe. In addition, these tests showed that cell permeabilization with low Proteinase K concentrations was sufficient for probe penetration.

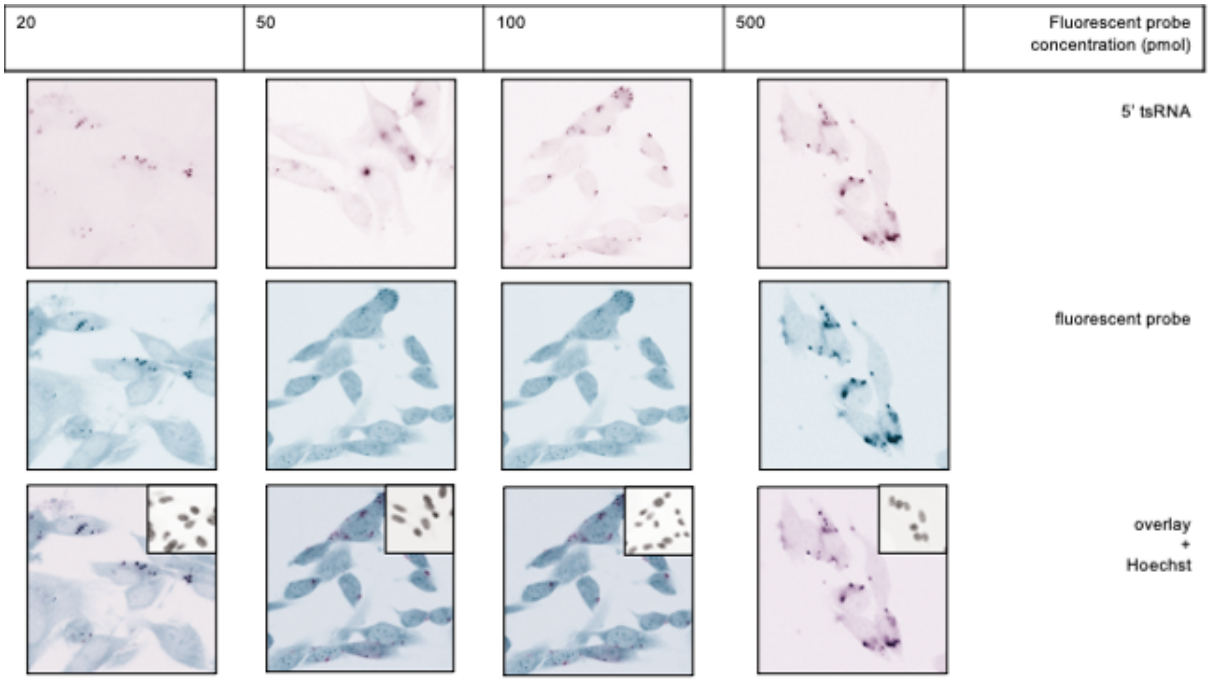


Figure 16. **Strength of co-localizing signal increases in intensity with increasing fluorescent probe concentration.**

MEF cells were grown on Geltrex-coated glass coverslips until confluent, then liposomally transfected with 2.5 µg of Atto590-labeled synthetic 5' tsRNA using DOTAP transfection reagent. The cells were kept in transfection media for 24 hours at 37 °C, afterwards fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K and incubated with indicated amounts of an Alexa488-labeled probe complementary to 5' tsRNA over-night. Analysis was performed by confocal microscopy. The results showed co-localization of 5' tsRNA-Gly^{GCC} and probe (depicted in purple in Figure 16). The abundance of co-localization signal increased with the amount of fluorescent probe applied. Distribution of the fluorescent probe signal indicated probe-binding to transfected 5' tsRNA and to parental tRNAs.

Next, it was tested if applying RtcB-mediated compLINK ligation followed by HCR resulted in discernable signal amplification on transfected 5' tsRNA-Gly^{GCC}. Signal amplification was expected to occur only upon hybridization of an HCR initiator-carrying compLINK, which was embedded in two overhangs on the compLINK (Figure 17A). As a control, cells subjected to all steps without initiator-hybridization were included.

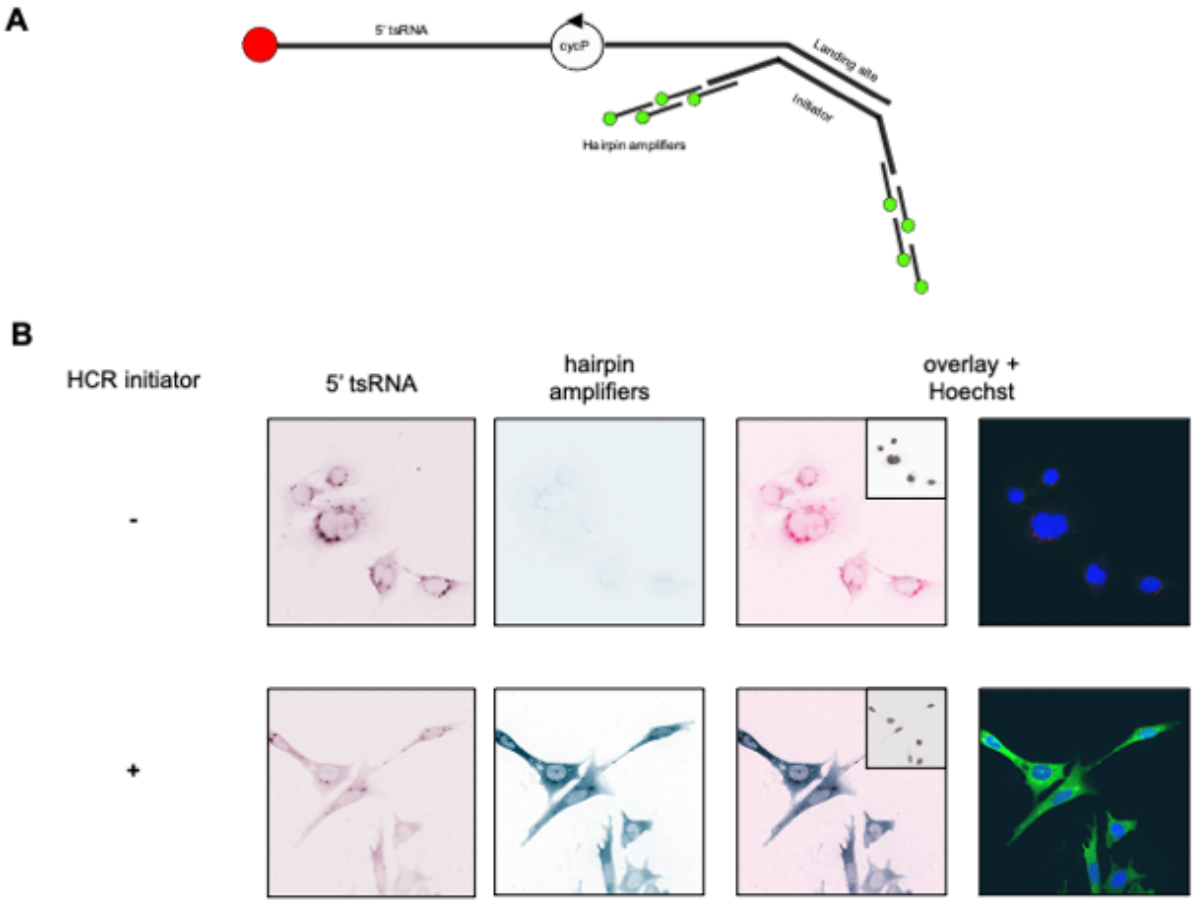


Figure 17. **Hybridization chain reaction is dependent on presence of an initiator sequence.**

(A) A compLINK complementary to 5' tsRNA is hybridized to the target. After successful hybridization the protruding landing site is hybridized to the HCR-initiating sequence. The two initiators, located one on each arm of the compLINK, trigger self-assembly of the alternating fluorescently labeled hairpin amplifiers. **(B)** MEF cells were grown on Geltrex-coated glass coverslips until confluent, then liposomally transfected with 2.5 µg of Atto590-labeled synthetic 5' tsRNA using DOTAP transfection reagent. The cells were kept in transfection media for 24 hours at 37 °C, afterwards fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K and incubated with 100 pmol of a compLINK over-night. Afterwards they were incubated with 100 pmol of an HCR initiating oligonucleotide over-night. Hairpin assembly for HCR was carried out with 12 pmol of each hairpin amplifier for two hours. Analysis was performed by confocal microscopy.

Analysis by confocal microscopy showed that assembly of hairpin amplifiers was specifically triggered by the presence of the compLINK containing the HCR initiator (Figure 17B). Co-localization of 5'tsRNA and hairpin amplifiers resulted in significant signal increase at dot-like areas where transfected 5' tsRNA-Gly^{GCC} accumulated. Unspecific HCR signals deviating from dot-like transfection signals indicated that hairpin amplifiers assembled also in areas where the concentration of 5' tsRNAs was lower or that the first hybridization with the compLINK was not exclusive to 5' tsRNAs but likely occurred also on tRNAs, thereby causing substantial background signal. Comparison of signal intensities between conventional

FISH and HCR-mediated amplification showed significantly higher signal yields when applying HCR (Figure 18). The HCR approach also resulted in higher accuracy of target signal amplification by initiating signals in most of the accumulated 5’ tsRNAs, while intensity of conventional FISH depended on amount of 5’ tsRNAs in their respective dot-like accumulation.

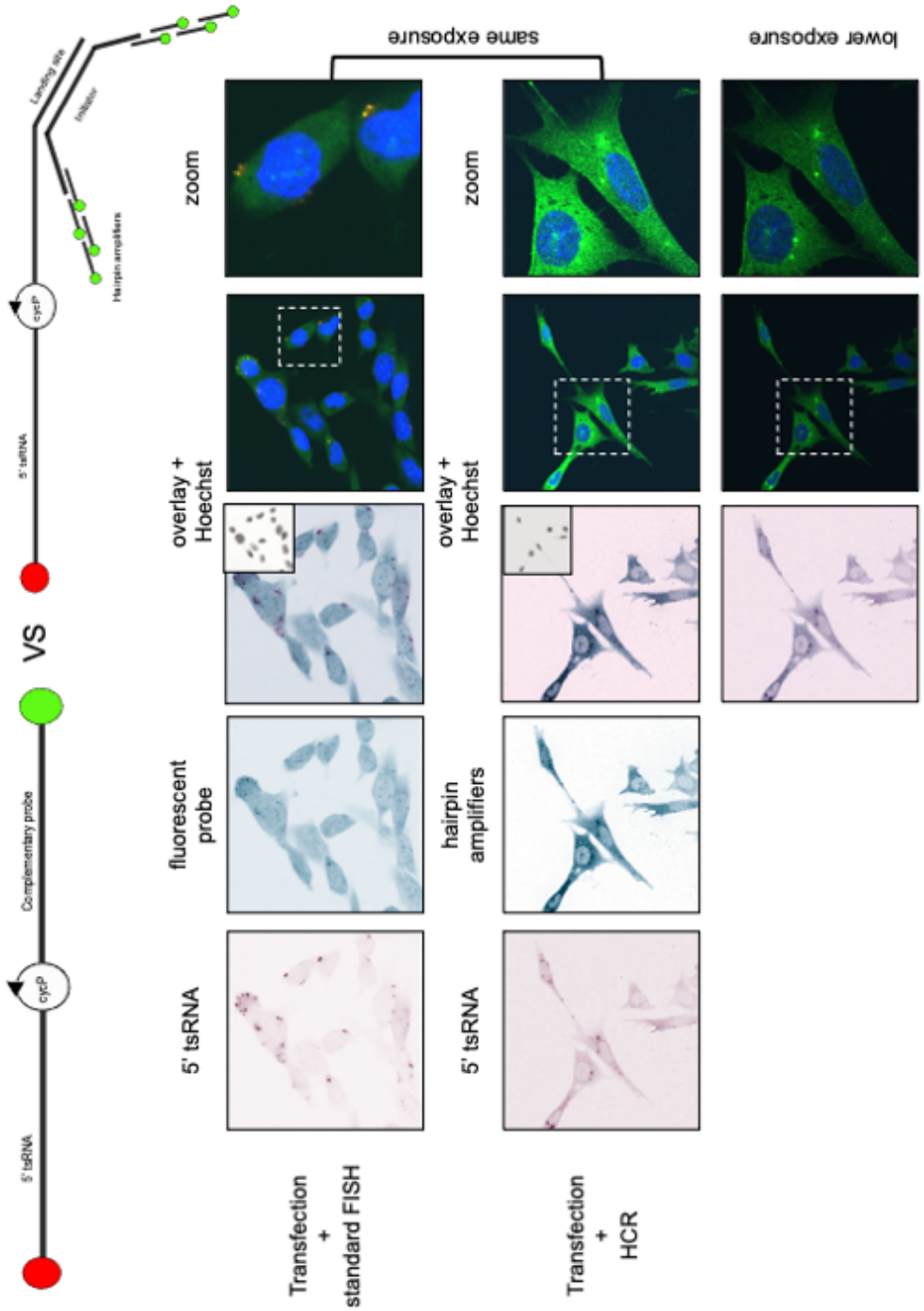


Figure 18. **HCR causes strong signal amplification with higher signal intensities than conventional FISH.**

The application of HCR led to stronger signal amplification and less unspecific background signal compared to a conventional fluorescence hybridization method. Single dots consisting of 5' tsRNAs that were liposomally transfected into MEF cells were selectively amplified with HCR, whereas with the conventional FISH method not every dot-like accumulation of transfected 5'tsRNA was co-localizing with the complementary probe.

5.2.3 Probe-stripping results in loss of background signals

Application of conventional FISH and of HCR-mediated signal amplification resulted in background signals, which were likely to be caused by unspecific binding of compLINKs containing either fluorescent moiety or HCR initiator sequences. In order to reduce these background signals, a stringent method for the removal of compLINKs that were not ligated to 5' tsRNAs but still hybridized to parental tRNA-Gly^{GCC} was required.

As described previously (Zhuang et al. 2020), high stringency washes with tetramethylammonium chloride (TMAC) were performed with the aim of removing background signals not originating from transfected 5' tsRNAs (Figure 19).

To test the effect of TMAC washes, different durations of the treatment were performed. Analysis by confocal microscopy revealed that TMAC did remove background signals to a certain extent but increasing the duration of the washes did not result in complete removal of background signals.

Simultaneously, another method for more efficient background signal removal was tried. As described in Hu et al. 2017, probe-stripping with high concentrations of formamide (FA) can be an effective means for the complete removal of FISH probes. To test the probe-stripping capacities of FA, different FA concentrations and treatment temperatures were applied in a FISH setting, where transfected fluorescently labeled 5' tsRNAs were hybridized to fluorescent compLINKs without RtcB ligation.

Analysis by confocal microscopy showed that stripping with FA was a highly effective method for stringently removing compLINKs. In respect to stripping temperature, no significant differences in probe-stripping efficiency could be detected between 70 and 80 °C (Figure 20A). At the same time, concentration of FA did affect probe-stripping efficiency since 70 % FA removed more probe signal than 50 % FA (Figure 20B).

Therefore, formamide-mediated probe-stripping was used instead of washes with TMAC in order to reduce background signals.

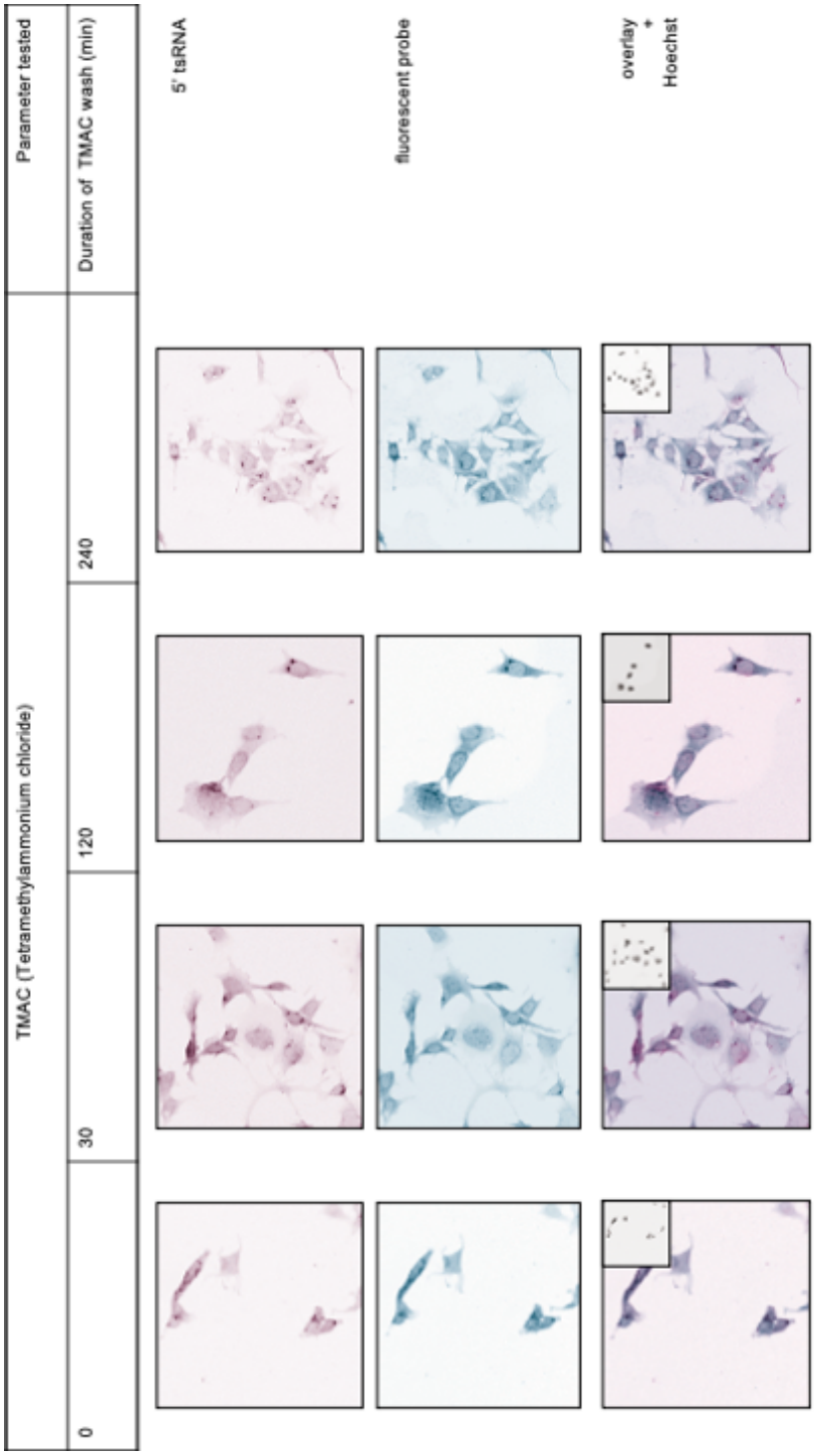


Figure 19. **Increasing duration of TMAC washes does not result in significant loss of background signals.**

MEF cells were grown on Vitronectin-coated glass coverslips until confluent, then liposomally transfected with 2.5 µg of Atto590-labeled synthetic 5' tsRNA using DOTAP transfection reagent. The cells were kept in transfection media for 24 hours at 37 °C, afterwards fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K and incubated with 100 pmol of an Alexa488-labeled probe complementary to 5' tsRNA over-night. High-stringency washes with TMAC were performed for indicated durations at 45 °C. Analysis was performed by confocal microscopy.

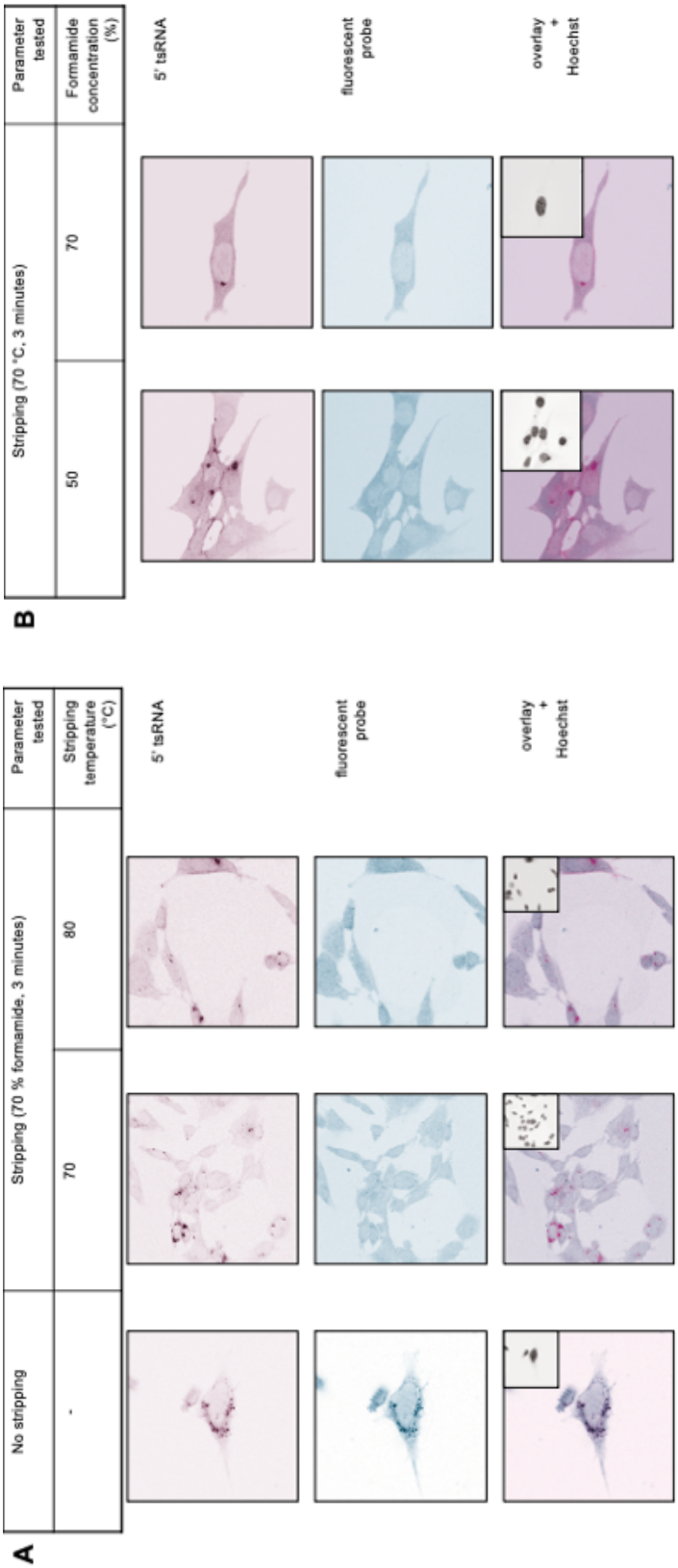


Figure 20. **Formamide-mediated probe-stripping effectively removes background signals.**

(A) MEF cells were grown on Laminin-coated glass coverslips until confluent, then liposomally transfected with 2.5 µg of Atto590-labeled synthetic 5' tsRNA using DOTAP transfection reagent. The cells were kept in transfection media for 24 hours at 37 °C, afterwards fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K and incubated with 100 pmol of an Alexa488-labeled probe complementary to 5' tsRNA over-night. Upon hybridization the coverslips were placed into 70% formamide for 3 minutes at 70 °C or 80 °C with the aim of removing unspecific background signal. **(B)** MEF cells were grown on Geltrex-coated glass coverslips until confluent, then liposomally transfected with 2.5 µg of Atto590-labeled synthetic 5' tsRNA using DOTAP transfection reagent. The cells were kept in transfection media for 24 hours at 37 °C, afterwards fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K and incubated with 100 pmol of an Alexa488-labeled probe complementary to 5' tsRNA over-night. Upon hybridization the coverslips were placed into 50% or 70% formamide for 3 minutes at 70 °C.

5.2.4 RtcB ligation prior to probe-stripping prevents loss of 5' tsRNA-specific signals

When incorporating the FA-based probe-stripping step into the FISH approach, the assumption is that RtcB-mediated ligation of compLINK to 5' tsRNA-Gly^{GCC} is a prerequisite for preventing the removal of specific compLINK-originating signal. Figure 20A and 20B show that not only background signals but also signals originating from compLINKs at sites of transfected 5' tsRNA-Gly^{GCC} were removed by stripping.

To address if RtcB-mediated ligation prevented signal removal of compLINKs at sites of transfected 5' tsRNA-Gly^{GCC}, different RtcB concentrations were tested to covalently link 5' tsRNAs to compLINK *in vivo* prior to probe-stripping. After ligation, formamide-mediated stripping was performed to evaluate the efficiency of removing background signals as well as the remaining signals originating from 5' tsRNAs (Figure 21).

The results showed a loss of background signals, leaving mainly fluorescent compLINK signal that co-localized with transfected 5' tsRNA-Gly^{GCC}. This application of conventional FISH also indicated that the signal intensity of remaining compLINK signal (linked to the 5' tsRNA targets) was rather weak suggesting that the yield of signal might not be sufficient for localizing smaller amounts of 5' tsRNAs. Regarding the concentration of RtcB, no significant differences in remaining background signals were detected, which is why high RtcB concentrations were selected for the following experiments.

Further parameter testing and method optimization was carried out using human-derived cancer cells.

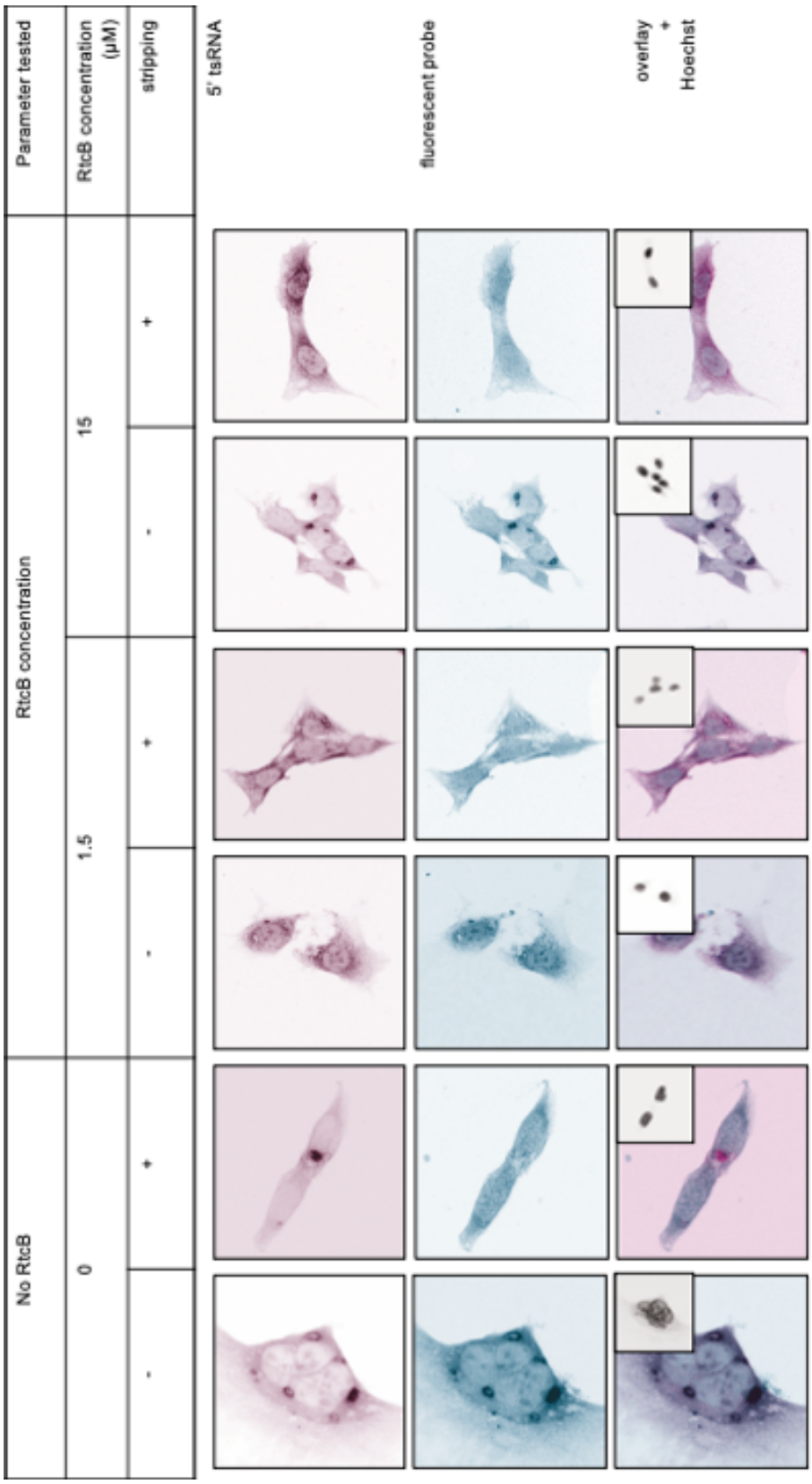


Figure 21. **RtcB ligation prevents stripping of covalently linked target and fluorescent probe.**

MEF cells were grown on Geltrex-coated glass coverslips until confluent, then liposomally transfected with 2.5 µg of Atto590-labeled synthetic 5' tsRNA using DOTAP transfection reagent. The cells were kept in transfection media for 24 hours at 37 °C, afterwards fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K and incubated with 100 pmol of an Alexa488-labeled probe complementary to 5' tsRNA over-night. Upon hybridization the coverslips were incubated over-night with indicated concentrations of RtcB ligase to covalently link 5'tsRNA and the fluorescent probe. For stripping of background signal the coverslips were placed into 70 % formamide for 3 minutes at 70 °C. Analysis was performed by confocal microscopy.

5.3 *In vivo* tests in human cancer cells

5.3.1 Optimal permeabilization of HeLa cells

Permeabilization of the sample and access of complementary probe to RNA target is a crucial step in every FISH protocol and is required to enable a certain level of penetration by the probe while not affecting the morphological intactness of the sample. To assess the optimal degree of compLINK permeability, different Proteinase K concentrations were tested for different durations in HeLa cells.

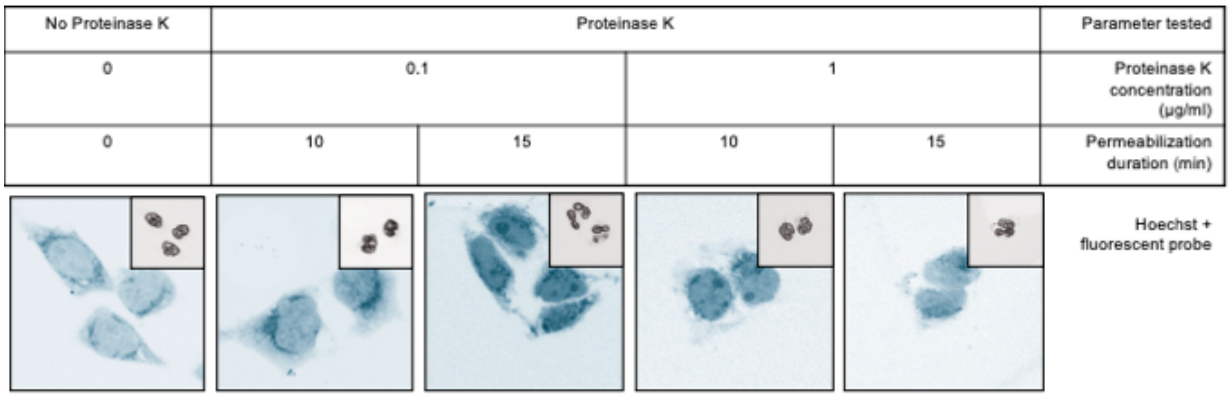


Figure 22. **Permeabilization with Proteinase K concentrations exceeding 0.1 µg/ml affect morphological integrity of HeLa cells.**

HeLa cells were grown on Geltrex-coated glass coverslips until confluent, afterwards fixed with 4 % PFA and permeabilized with 0.1 or 1 µg/ml Proteinase K and incubated with 100 pmol of an Alexa488-labeled probe complementary to 5' tsRNA over-night. Analysis was performed by confocal microscopy.

The results showed that increasing duration and concentration of Proteinase K treatment resulted in cell detachment from coverslips as well as disruption of cellular morphology (Figure 22). In addition, treatment with higher concentrations of Proteinase K did not result in increased abundance of compLINKs within cells. In contrast, lower Proteinase K concentrations did not affect intactness of the cells and were sufficient to enable probe penetration without disrupting cells or risk of cell loss, which was consistent with previous results obtained in MEF cells. Therefore, treatment with 0.1 µg/ml Proteinase K for 10 minutes was applied for all following experiments.

5.3.2 Optimization of stripping conditions

To test if efficiency of stripping with FA can be increased, the composition of the applied probe-stripping buffer was modified by addition of saline sodium citrate (SSC). It was also tested if the duration of probe-stripping and post-stripping treatments influenced the persistence of background signals within cells.

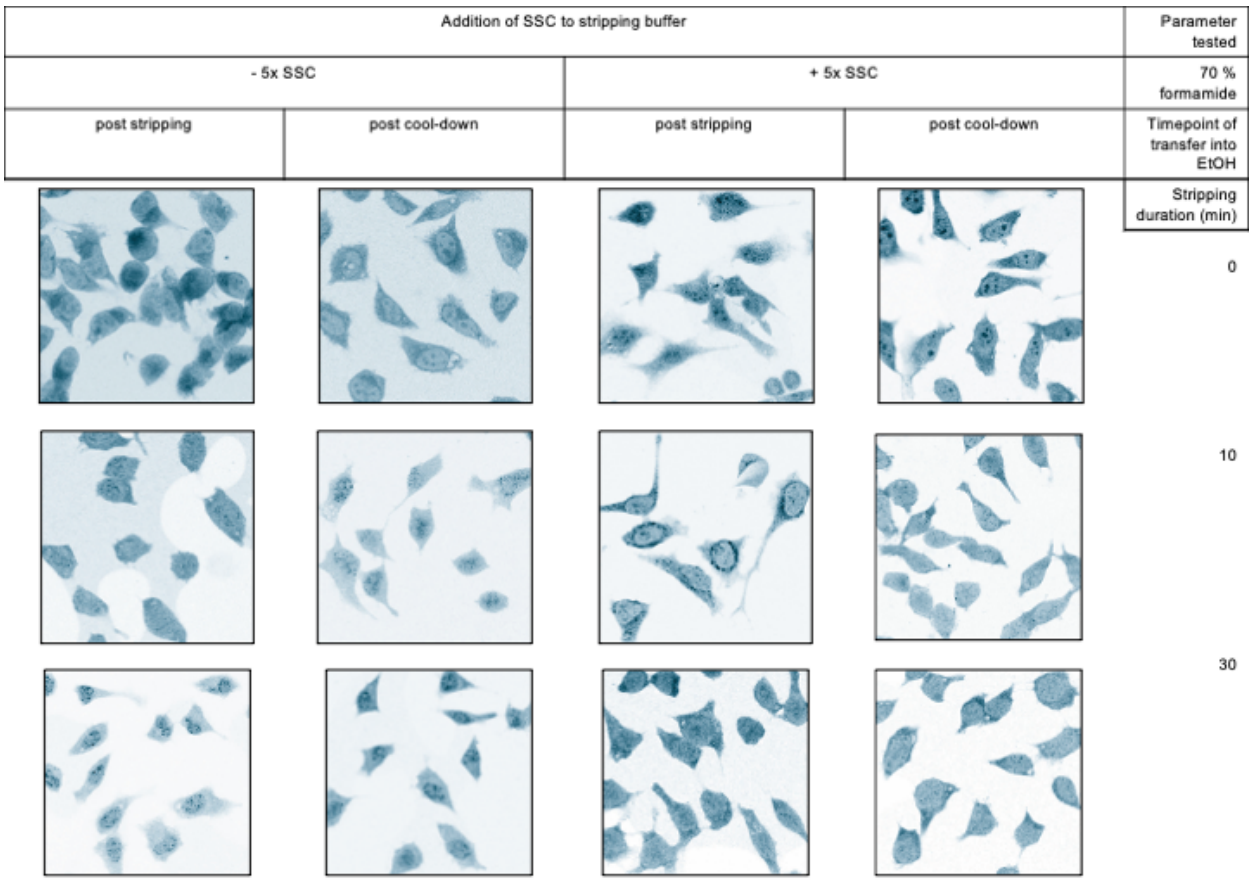


Figure 23. **Modified stripping buffer composition results in higher stripping efficiency.**

HeLa cells were grown on Geltrex-coated glass coverslips until confluent, afterwards fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K and incubated with 100 pmol of a compLINK over-night. After this first hybridization 70 % formamide stripping at 70°C with or without addition of 5x SSC was performed for 10 or 30 minutes. Upon stripping coverslips were either transferred into ice-cold 75% EtOH or placed at room temperature for 30 minutes and transferred into ethanol afterwards. Subsequently, coverslips were incubated with 100 pmol of an HCR-initiating oligonucleotide over-night. Hairpin assembly for HCR was carried out with 12 pmol of each hairpin amplifier for two hours. Analysis was performed by confocal microscopy.

Analysis by confocal microscopy showed different distribution patterns of background signals, which were dependent on the addition of 5x SSC and the duration of the probe-stripping step prior to transfer to ethanol. Without addition of 5x SSC to the FA-stripping buffer, the background signals were comparable between samples that were either transferred directly to ethanol or after a cool-down phase. The latter samples showed a great amount of swollen and ruptured cells, which was likely due to an

extended incubation period (cool-down phase) in FA. HCR-amplified signals were mainly dispersed as speckles in the nucleus. In contrast, the addition of 5x SSC to the FA-stripping buffer resulted in removal of nuclear speckle-like signals and overall weaker signals in the cytoplasm. Direct transfer to ethanol was more effective than extended cooling down in FA-stripping buffer, while there was no significant difference in removal of signals between 10- and 30-minutes probe-stripping treatment (Figure 23). These results indicated that addition of 5x SSC to the FA-stripping buffer improved compLINK stripping efficiency, especially when followed by transfer to ethanol directly after stripping treatment.

Furthermore, it was assessed if probe-stripping buffer exchange after fifteen minutes influenced compLINK stripping efficiency or if treatment with a lower FA concentration was more efficient than treatment with ethanol. The results of these protocol variations were analyzed by tracking of the HCR-amplified compLINK signals.

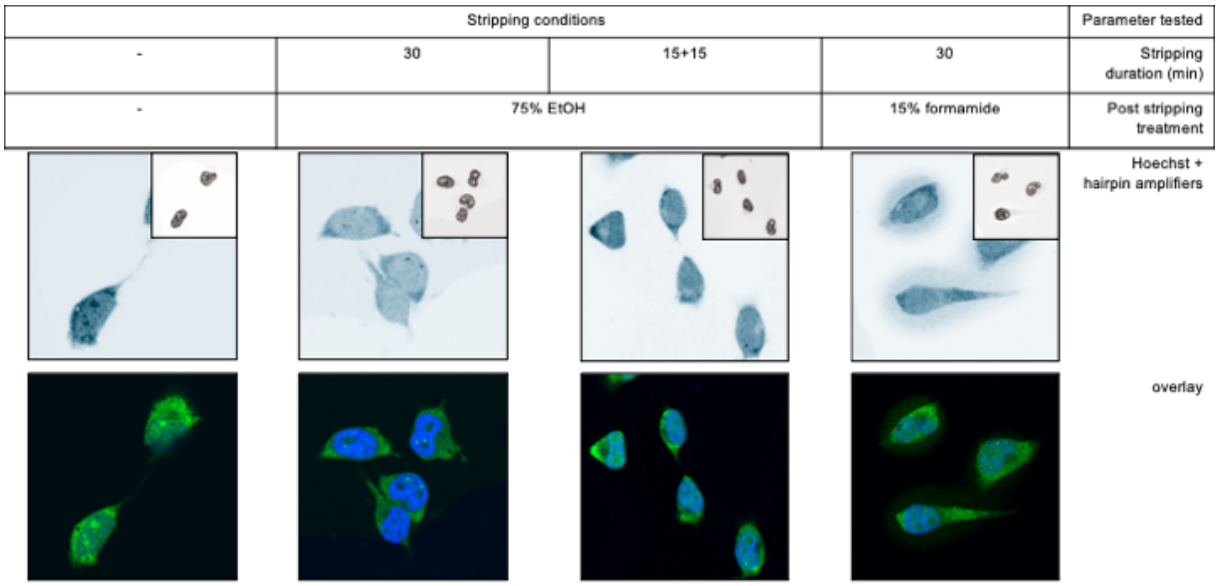


Figure 24. **Stripping efficiency is not improved by buffer exchange or extended FA treatment.**

HeLa cells were grown on Geltrex-coated glass coverslips until confluent, afterwards fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K and incubated with 100 pmol of a compLINK over-night. Afterwards stripping with 70 % formamide + 5x SSC at 70 °C with or without exchange of stripping buffer after 15 minutes was performed for 30 minutes in total. After stripping coverslips were either transferred into ice-cold 75% EtOH or washed with 15% formamide three times for 10 minutes at room temperature and transferred into ethanol afterwards. Subsequently, coverslips were incubated with 100 pmol of an HCR initiating oligonucleotide over-night. Hairpin assembly for HCR was carried out with 12 pmol of each hairpin amplifier for two hours. Analysis was performed by confocal microscopy.

The results showed that washing with lower FA concentrations after the initial probe-stripping treatment did not result in improved background signal removal, neither did exchanging the FA-stripping buffer after 15 minutes (Figure 24). Compared to the distribution of background signals resulting from other treatments, compLINK stripping constantly for 30 minutes followed by an ethanol wash remained to be the most efficient method for the efficient removal of background signals.

Another parameter that was tested for the removal of background signals was an incubation step with a toehold-probe during probe stripping. Toehold probes were intended to interfere with the hybridization of compLINKs to parental tRNAs after the probe-stripping step, thereby preventing HCR-mediated signal amplification of background signals.

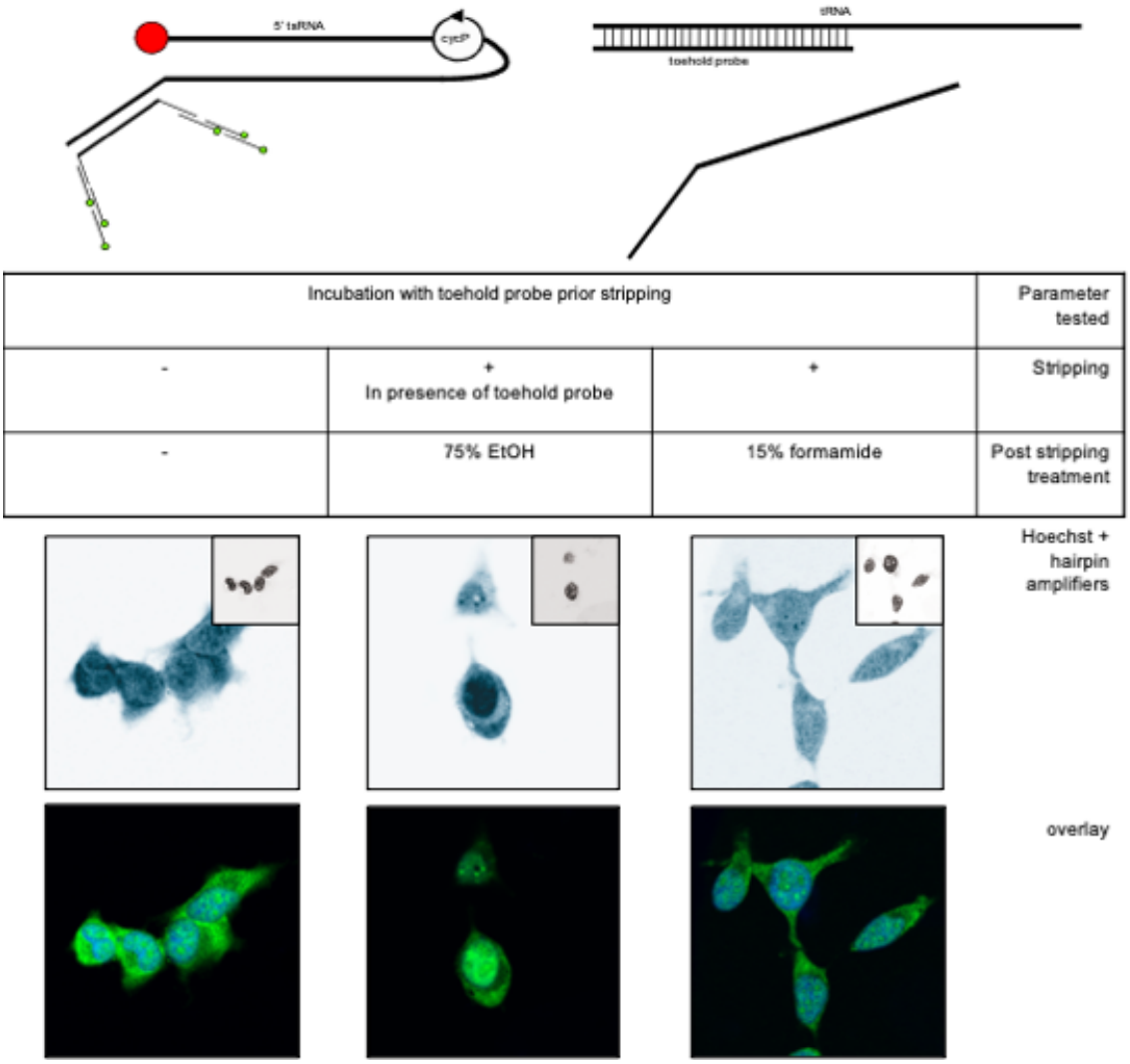


Figure 25. **Presence of a toehold-probe during stripping does not block background signal.**

HeLa cells were grown on Geltrex-coated glass coverslips until confluent, afterwards fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K and incubated with 100 pmol of a compLINK over-night. Incubation with 100 pmol of a toehold-probe followed for 30 minutes at 37 °C. Afterwards stripping with 70 % formamide + 5x SSC at 70 °C with or without addition of the toehold-probe to the stripping buffer was performed for 30 minutes. After stripping coverslips were either transferred into ice-cold 75% EtOH or washed with 15 % formamide three times for 10 minutes at room temperature and transferred into ethanol afterwards. Subsequently, coverslips were incubated with 100 pmol of an HCR initiating oligonucleotide over-night. Hairpin assembly for HCR was carried out with 12 pmol of each hairpin amplifier for two hours. Analysis was performed by confocal microscopy.

Confocal analysis showed that inclusion of a toehold-probe prior during stripping did not result in lower background signals indicating that blocking re-hybridization of non-ligated compLINKs with parental tRNAs was not effective. In addition, washes with 15 % FA after probe-stripping did not result in better background removal either. Curiously, inclusion of a toe-hold probe during probe-stripping even resulted in stronger HCR amplification signals (Figure 25).

5.3.3 Optimization of HCR

To test if the signal yield resulting from HCR-mediated amplification could be further increased, different parameters were varied. In particular, it was tested if an increased duration of the HCR amplification cycle or an increase in hairpin amplifier concentration would yield a stronger amplification signal.

Confocal analysis showed that increasing the duration of the HCR amplification step resulted in stronger hairpin amplifier signals, especially in dot-like accumulations at sites of transfected 5' tsRNA-Gly^{GCC} (Figure 26). Compared to two hours of HCR amplification, the amplified signals from four hours of HCR amplification resulted in a substantial signal increase, whereas the signals from 18 hours HCR amplification did not differ significantly from those of the four-hour HCR cycle. Notably, providing higher concentrations of hairpin amplifiers also resulted in increased signals both after four and 18 hours of HCR amplification. Importantly, signals resulting from 18 hours of HCR amplification did not increase proportionally when compared to the 4 hours HCR cycle.

As previously observed, HCR signal amplification resulted in background signal amplification, which was also to be expected in case of longer reaction cycles with higher hairpin amplifier concentrations. For that reason, the experiment was repeated including the probe-stripping step, with previously experimentally determined optimized stripping conditions.

The results showed that strongly amplified HCR signals co-localized with dot-like accumulation of transfected 5' tsRNA-Gly^{GCC} and resulted in bright yellow spots when merged (Figure 27). This applied especially to four- and 18 hours HCR cycles. Higher concentrations of hairpin amplifiers also showed increased HCR amplification signals for four or 18 hours of HCR cycles. Again, signals from 18 hours of HCR did not increase proportionally when compared to the 4 hours cycle, which was consistent with previous results. Background signal removal by probe-stripping revealed to be more efficient after two or four hours of HCR amplification using 12 or 24 pmol of each hairpin amplifier.

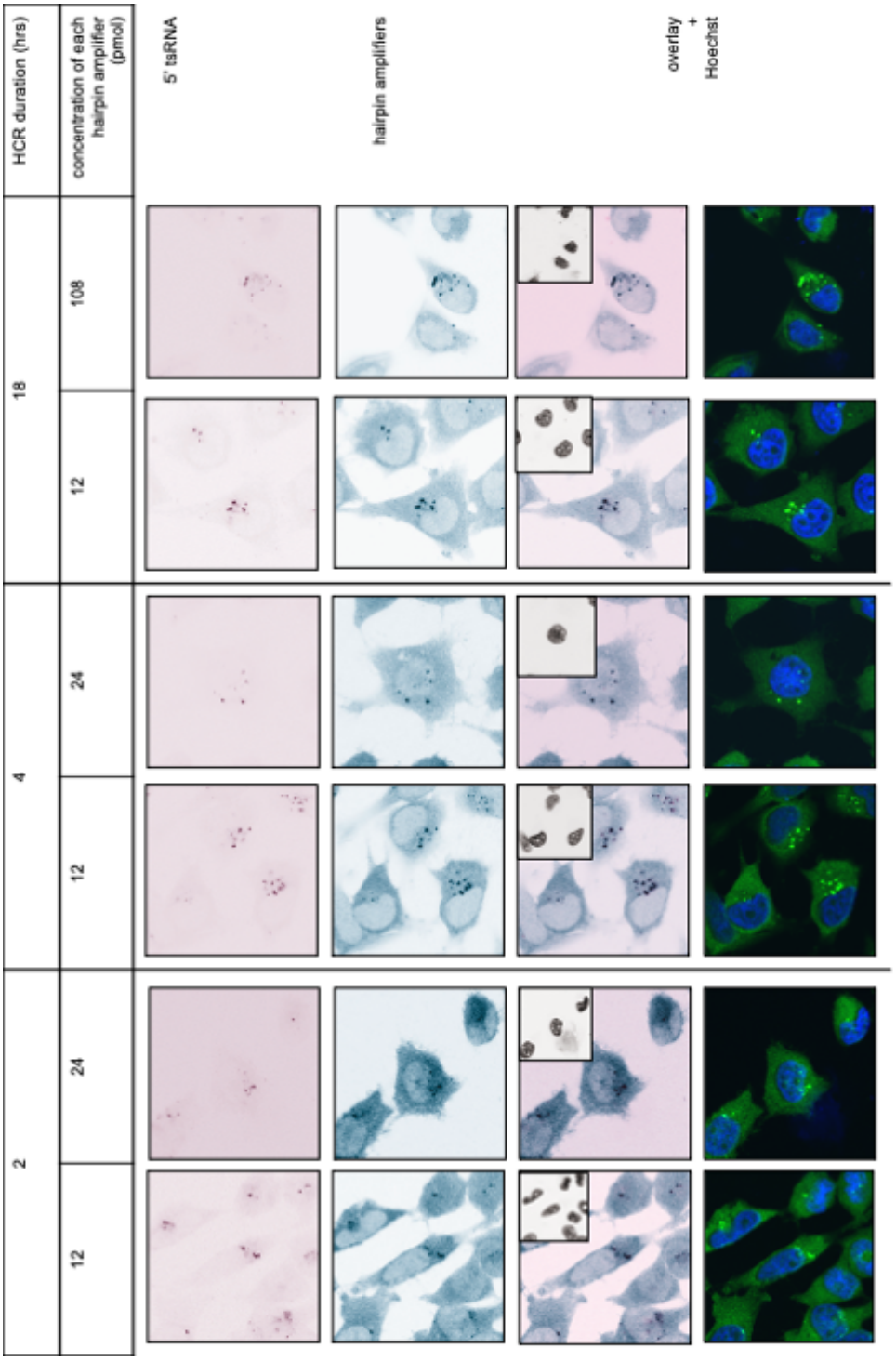


Figure 26. **Extension of HCR duration results in higher signal yield.**

HeLa cells were grown on Geltrex-coated glass coverslips until confluent, then liposomally transfected with 2.5 µg of Atto590-labeled synthetic 5' tsRNA using DOTAP transfection reagent. The cells were kept in transfection media for 24 hours at 37 °C, afterwards fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K and incubated with 100 pmol of a compLINK over-night. Subsequently, coverslips were incubated with 100 pmol of an HCR initiating oligonucleotide over-night. Hairpin assembly for HCR was carried out with indicated amounts of each hairpin amplifier for two, four and 18 hours. Analysis was performed by confocal microscopy.

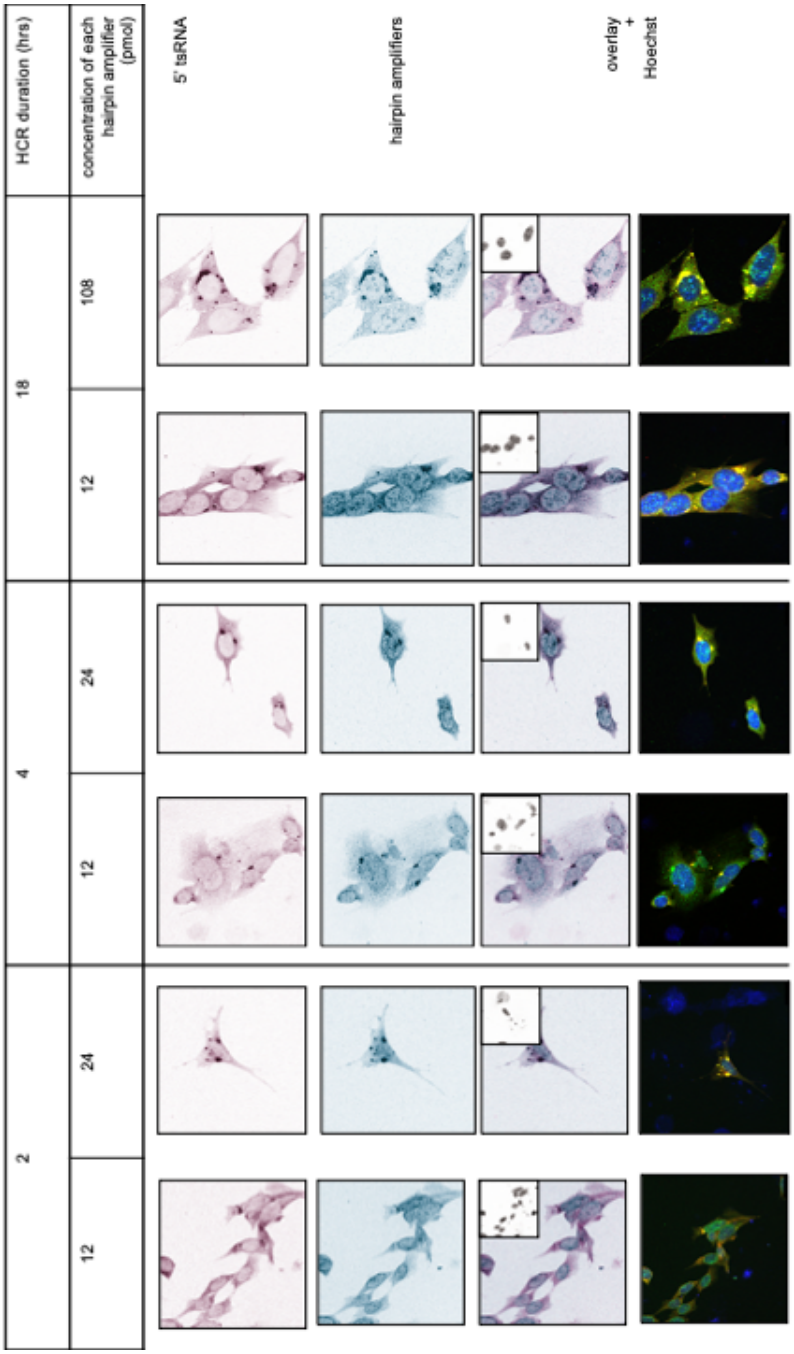


Figure 27. **HCR amplification selectivity increases upon RtcB ligation followed by probe-stripping.**

HeLa cells were grown on Geltrex-coated glass coverslips until confluent, then liposomally transfected with 2.5 µg of Atto590-labeled synthetic 5' tsRNA using DOTAP transfection reagent. The cells were kept in transfection media for 24 hours at 37 °C, afterwards fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K and incubated with 100 pmol of a compLINK over-night. Upon hybridization the coverslips were incubated over-night with 35 µM RtcB ligase to covalently link 5'tsRNA and compLINK. For stripping of background signal the coverslips were placed into 70 % formamide + 5x SSC at 70 °C for 30 minutes and placed into ice-cold 75% EtOH afterwards. Subsequently, coverslips were incubated with 100 pmol of an HCR initiating oligonucleotide over-night. Hairpin assembly for HCR was carried out with indicated amounts of each hairpin amplifier for two, four and 18 hours. Analysis was performed by confocal microscopy.

5.3.4 Testing HCR 3.0

With the aim of making HCR amplification signals even more specific for specific 5' tsRNAs while reducing the burden of technical manipulation of samples (i.e., through harsh probe-stripping), further adjustments to the compLINK design were made. To this end, the HCR initiator sequence was split into two halves (as published for HCR 3.0 by Choi et al. 2018) whereby one half was linked to a probe complementary to the 5' tsRNA-Gly^{GCC} and the other half to a probe with complementary to a hairpin-compLINK (Figure 2C). Furthermore, different hairpin amplifier designs were tested for signal amplification.

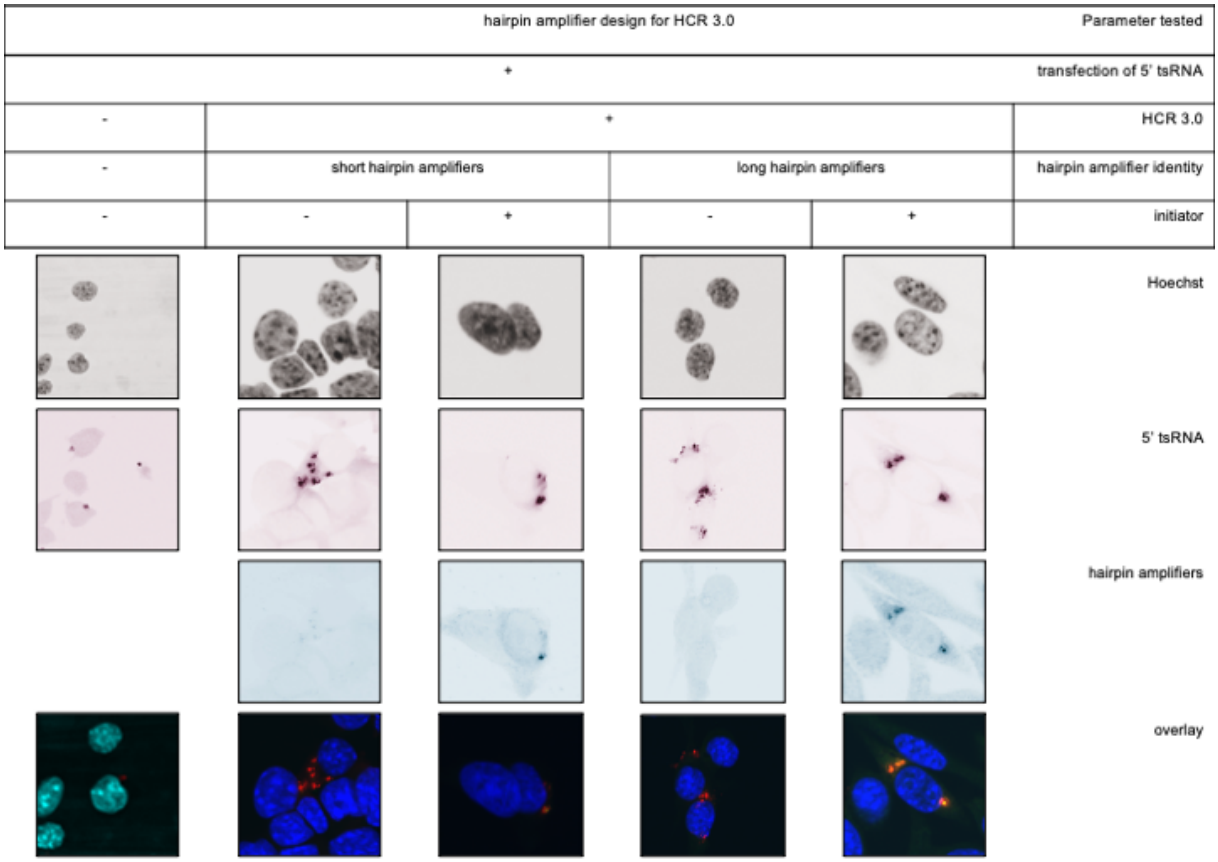


Figure 28. **Long hairpin amplifiers show higher signal intensity than short hairpin amplifiers.**

HeLa cells were grown on Laminin-coated glass coverslips until confluent and transfected with 2.5 µg of Atto590-labeled synthetic 5' tsRNA using DOTAP transfection reagent. Afterwards cells were fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K. For the first hybridization coverslips were incubated with 100 pmol of a hairpin-compLINK over-night. Upon hybridization the coverslips were incubated over-night with 35 µM RtcB ligase to covalently link 5' tsRNA and the hairpin-compLINK. In the next step the cells were incubated with a split initiator probe pair, with a concentration of 1.2 pmol for each split initiator. Hairpin assembly for HCR was carried out with 18 pmol of each hairpin amplifier for 18 hours. Analysis was performed by confocal microscopy.

The results showed that for both hairpin-designs no HCR amplification signal was triggered without previous incubation of samples with the split initiator probes. Including an incubation with split initiator probes prior to HCR amplification resulted in highly specific signals for hairpin-compLINKs (Figure 28),

which were co-localizing with signals from transfected 5’ tsRNA-Gly^{GCC}. Notably, use of longer hairpin amplifiers resulted in stronger HCR amplification signals than when using of short hairpin amplifiers.

Comparison of previous HCR approaches and HCR 3.0 revealed that the signal amplified by HCR 3.0 was exclusively located at sites of transfected 5’ tRNA-Gly^{GCC}. However, overall less intense fluorescent signal yield was detectable when compared to previous HCR approaches, which suggested that falsely triggered chain reactions of hairpin amplifier signal could be prevented by applying HCR 3.0 (Figure 2C) to RtcB-mediated ligation of specific 5’ tsRNAs to hairpin-compLINKs (Figure 29).

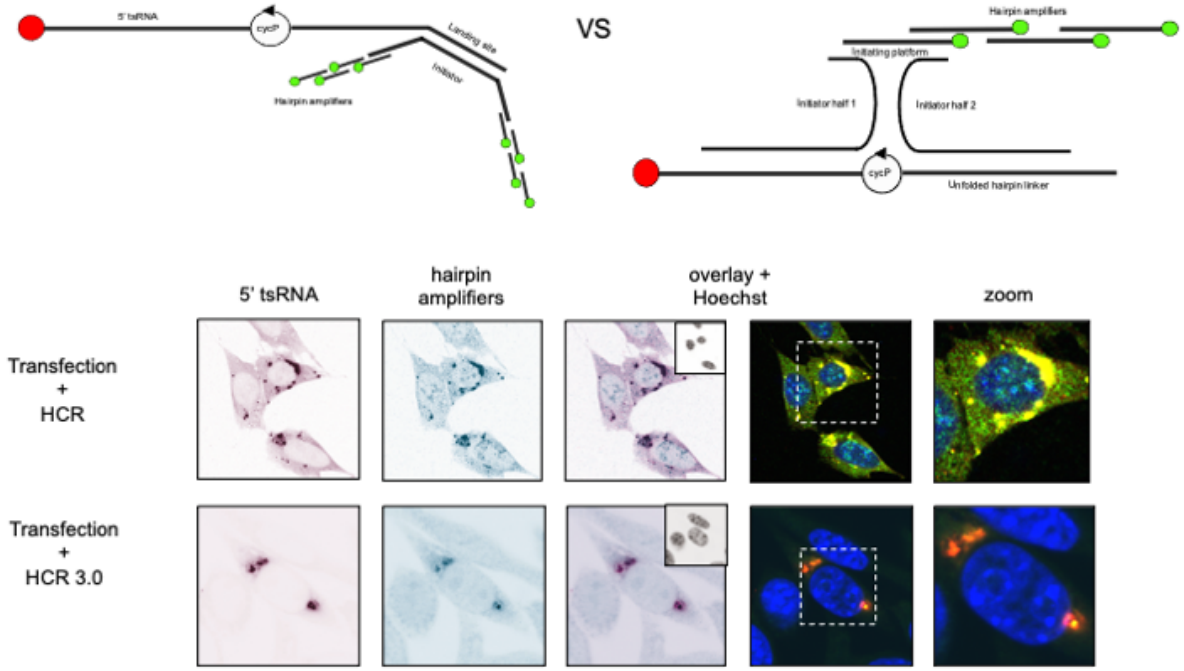


Figure 29. **Comparison of HCR versus HCR 3.0.**

Application of HCR results in strong signal amplification of transfected 5’ tsRNAs and unspecific background signal. Compared to previously used HCR the application of HCR 3.0 results in less strong signal amplification of transfected 5’ tsRNAs but does not amplify background sourcing from tRNAs, due to the split initiator’s higher selectivity for the target sequences.

5.3.5 Testing transfection parameters

To evaluate how long 5’ tsRNAs can be tracked after transfection, HeLa cells were transfected with 5’ tsRNA-Gly^{GCC} and observed for seven days post-transfection. The presence of 5’ tsRNA-Gly^{GCC} was determined by application of conventional FISH (Figure 2A) using RtcB-mediated ligation of compLINKs and the established probe-stripping protocols.

The results showed that 5’ tsRNAs could be tracked until the fifth day after transfection and that they mainly localized around the nucleus. Importantly, fluorescent signals from compLINKs decreased with increasing time after transfection, which indicated decreasing amounts of transfected 5’ tsRNAs over time (Figure 30).

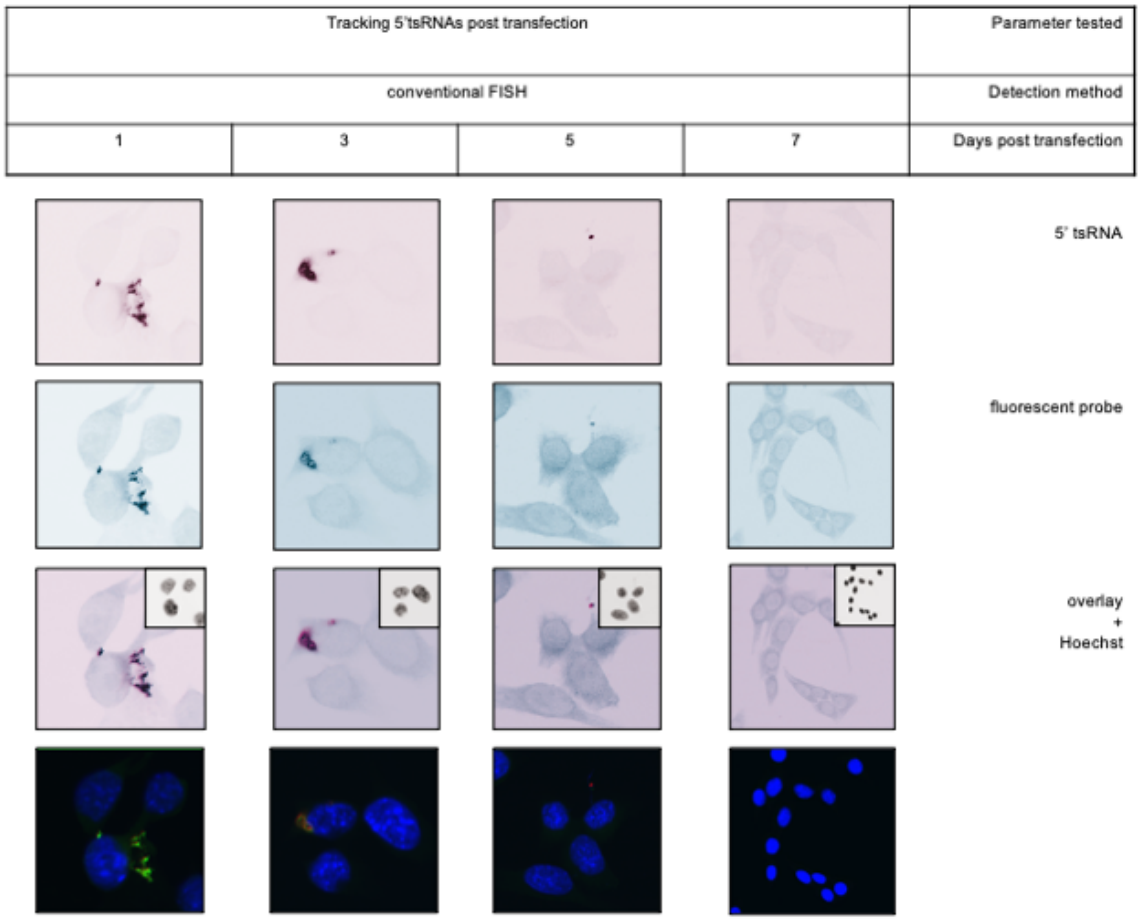


Figure 30. **Signal intensity from 5' tsRNAs as well as from fluorescent compLINKs decrease over time.**

HeLa cells were transfected with 2.5 µg of Atto590-labeled synthetic 5’ tsRNA using DOTAP transfection reagent. Transfected cells were transferred to Laminin-coated glass coverslips and upon attachment fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K. Cells were incubated with 100 pmol of an Alexa488-labeled complementary probe over-night. Upon hybridization the coverslips were incubated over-night with 35 µM RtcB ligase to covalently link 5’ tsRNA and the fluorescent probe. For stripping of background signal the coverslips were placed into 70 % formamide + 5x SSC at 70 °C for 30 minutes and placed into ice-cold 75 % EtOH afterwards. Analysis was performed by confocal microscopy.

To test the minimal amount of tsRNAs that was required for robust visualization by HCR amplification, different amounts of synthetic 5' tsRNA-Gly^{GCC} were transfected into HeLa cells followed by the detection through HCR-mediated amplification without RtcB ligation and probe-stripping. For assessment of transfection efficiency every coverslip contained approximately 1.2 x 10^6 HeLa cells.

Confocal microscopy showed that formation of dot-like accumulations of transfected 5' tsRNAs were more abundant in cells that had been transfected with higher amounts of 5' tsRNA-Gly^{GCC}. HCR amplification signals increased proportionally with increasing amounts of transfected 5' tsRNAs. Cells that were transfected with 1500 ng 5' tsRNAs triggered the strongest HCR amplification signal. However, co-localization of both signals was detected even with transfected amounts as little as 1.5 ng per 1.2 x 10^6 cells (Figure 31).

To compare the transfection efficiency when using DOTAP with Lipofectamine, transfected synthetic 5' tsRNA-Gly^{GCC} were detected by both conventional FISH and HCR.

The results showed that transfection of the same amount of 5' tsRNA-Gly^{GCC} resulted in different signal localization and intensity depending on the transfection reagent. Transfection by Lipofectamine caused bigger dot-like accumulations of transfected 5' tsRNAs in cells but also on coverslip surfaces, while transfection by DOTAP mainly delivered 5' tsRNAs into cells but with considerably fewer dots per cell. Signals from fluorescent compLINKs (Figure 2A) co-localized with 5' tsRNA-Gly^{GCC} signals, although signal amplification by HCR (Figure 2B) resulted in stronger signals. Co-localization areas were more abundant in cells transfected with Lipofectamine. Additionally, transfection with Lipofectamine caused fewer background signals within cells both in conventional FISH as well as in HCR approaches (Figure 32).

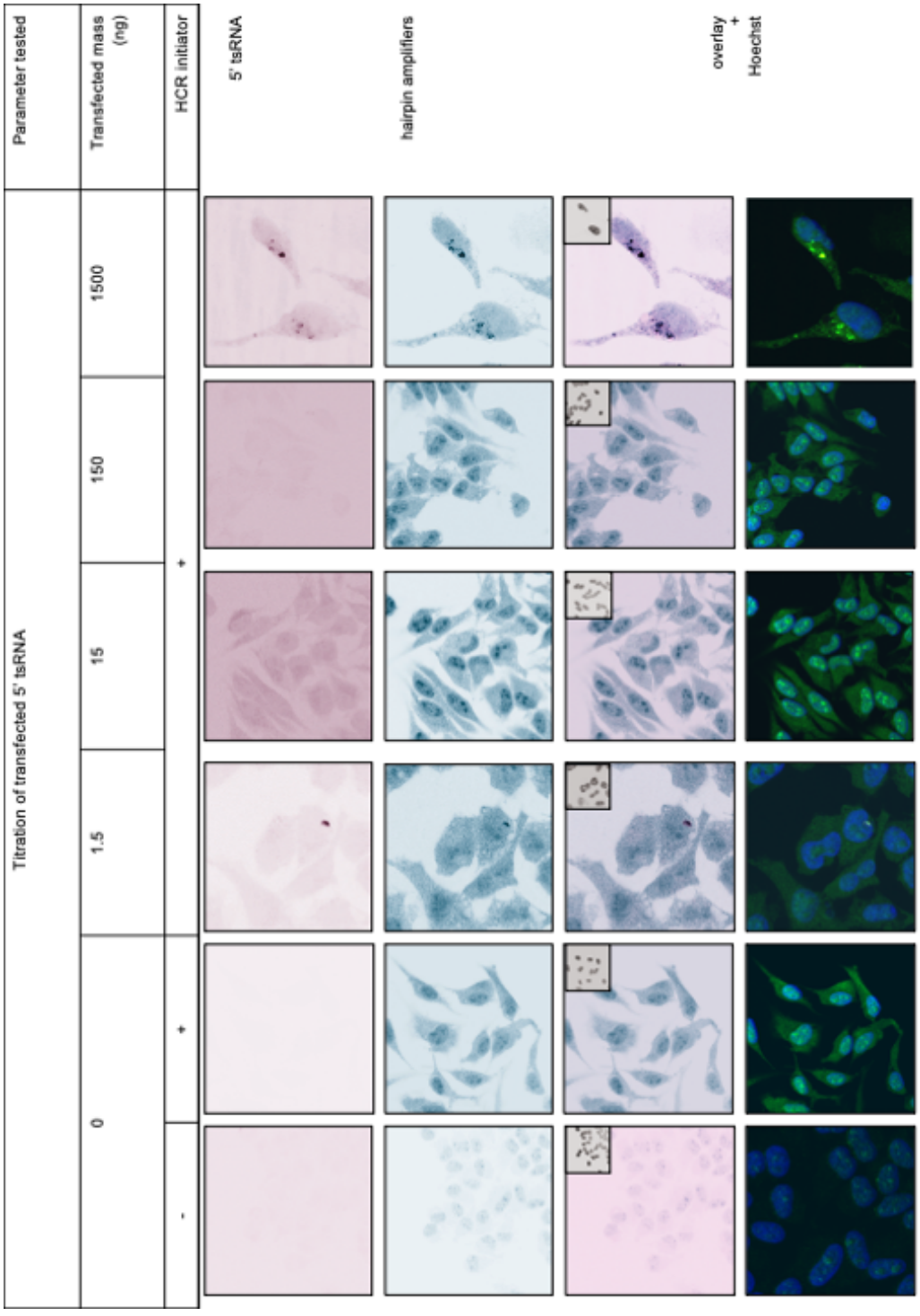


Figure 31. **Transfection of decreasing amounts of tsRNA results in weaker HCR signal amplification.**

HeLa cells were grown on Vitronectin-coated glass coverslips until confluent and transfected with indicated amounts of Atto590-labeled synthetic 5' tsRNA using DOTAP transfection reagent. Afterwards the cells were fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K. Cells were incubated with 100 pmol of a compLINK over-night. Subsequently, coverslips were incubated with 100 pmol of an HCR initiating oligonucleotide over-night. Hairpin assembly for HCR was carried out with 24 pmol of each hairpin amplifier for four hours. Analysis was performed by confocal microscopy.

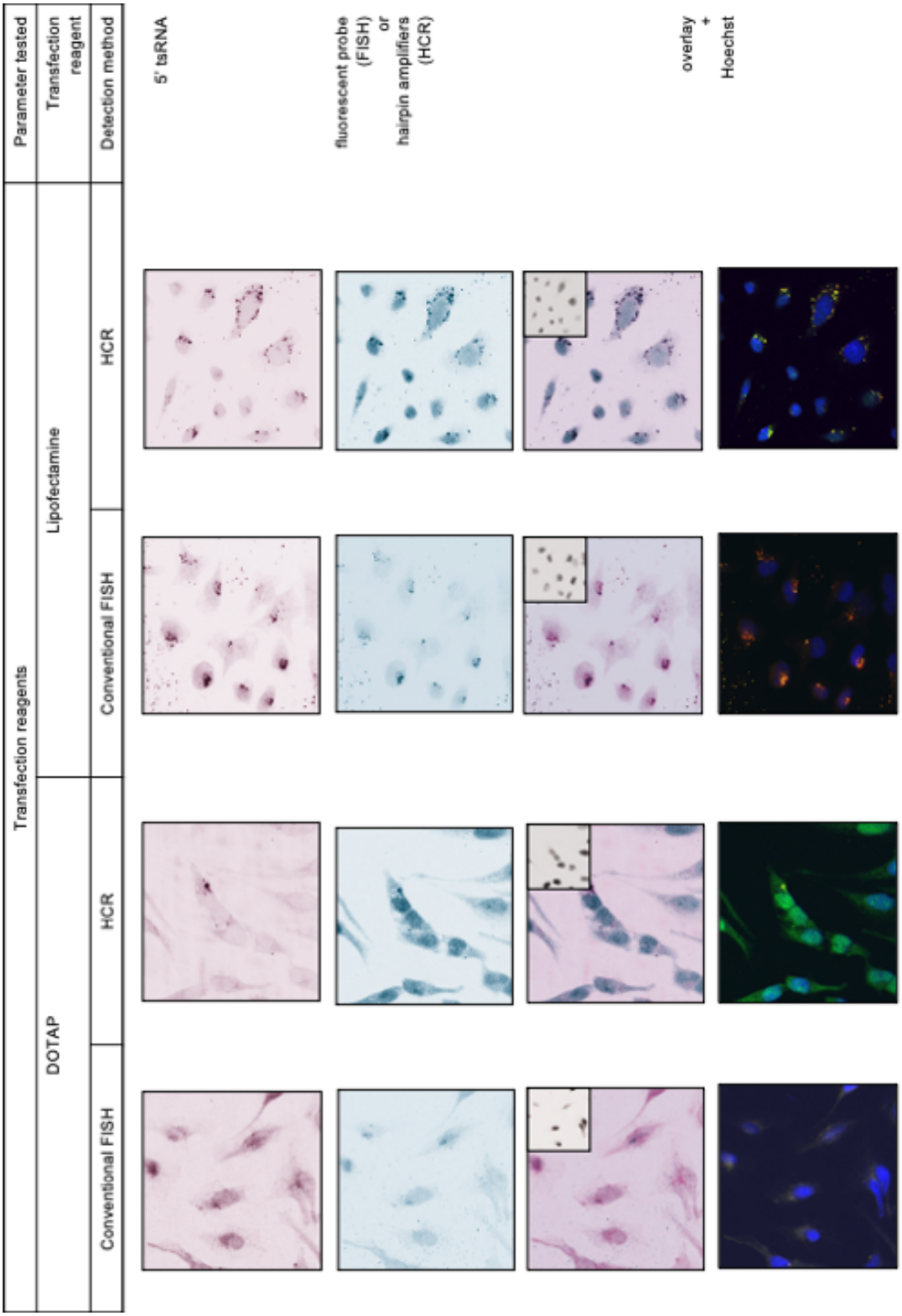


Figure 32. **Transfection with lipofectamine causes fewer background signals in both visualization approaches.**

HeLa cells were grown on Vitronectin-coated glass coverslips until confluent and transfected with 1.5 µg of Atto590-labeled synthetic 5' tsRNA using DOTAP and Lipofectamine transfection reagent. Afterwards the cells were fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K. Cells were incubated with 100 pmol of an Alexa488-labeled complementary probe (FISH) or with a compLINK (HCR) over-night. Subsequently, coverslips for HCR signal amplification were incubated with 100 pmol of an HCR initiating oligonucleotide over-night. Hairpin assembly for HCR was carried out with 24 pmol of each hairpin amplifier for four hours. Analysis was performed by confocal microscopy.

5.3.6 Inducing tRNA fragmentation with arsenite

To determine the limitations of the *in vivo* visualization of 5' tsRNAs, the abundance of endogenously produced 5' tsRNAs compared to synthetic 5' tsRNAs that were transfected into cells was assessed. To produce endogenous levels of 5' tsRNAs, HeLa cells were exposed to acute oxidative stress (Figure 33A) or exposed to and recovered from (for 24 hours) oxidative stress (Figure 33B) using inorganic sodium arsenite (iAs). For transfection, HeLa cells were transfected with different concentrations of synthetic 5' tsRNA-Gly^{GCC}. tRNA fragmentation was determined by northern blotting using a ^{32}P-radiolabelled complementary DNA probe against the 5' part of tRNA-Gly^{GCC}, which allowed assessing the relative quantities of endogenous tsRNAs (induced by iAs) compared to transfected 5' tsRNAs.

The results showed that acute iAs stress resulted in induction of tRNA fragmentation (Figure 33A). The intensity of the probe signal indicated that the amount of 5' tsRNA-Gly^{GCC} that was extracted from approximately 0.6 x 10^6 cells was comparable to transfections of approximately 1.2 x 10^6 cells with 15 to 150 ng of synthetic 5' tsRNA-Gly^{GCC}. Recovery from oxidative stress also resulted in tRNA fragmentation, but levels of 5' tsRNA-Gly^{GCC} did not increase as much compared to acute stress exposure and could not be as clearly quantified as in acutely stressed cells (33B).

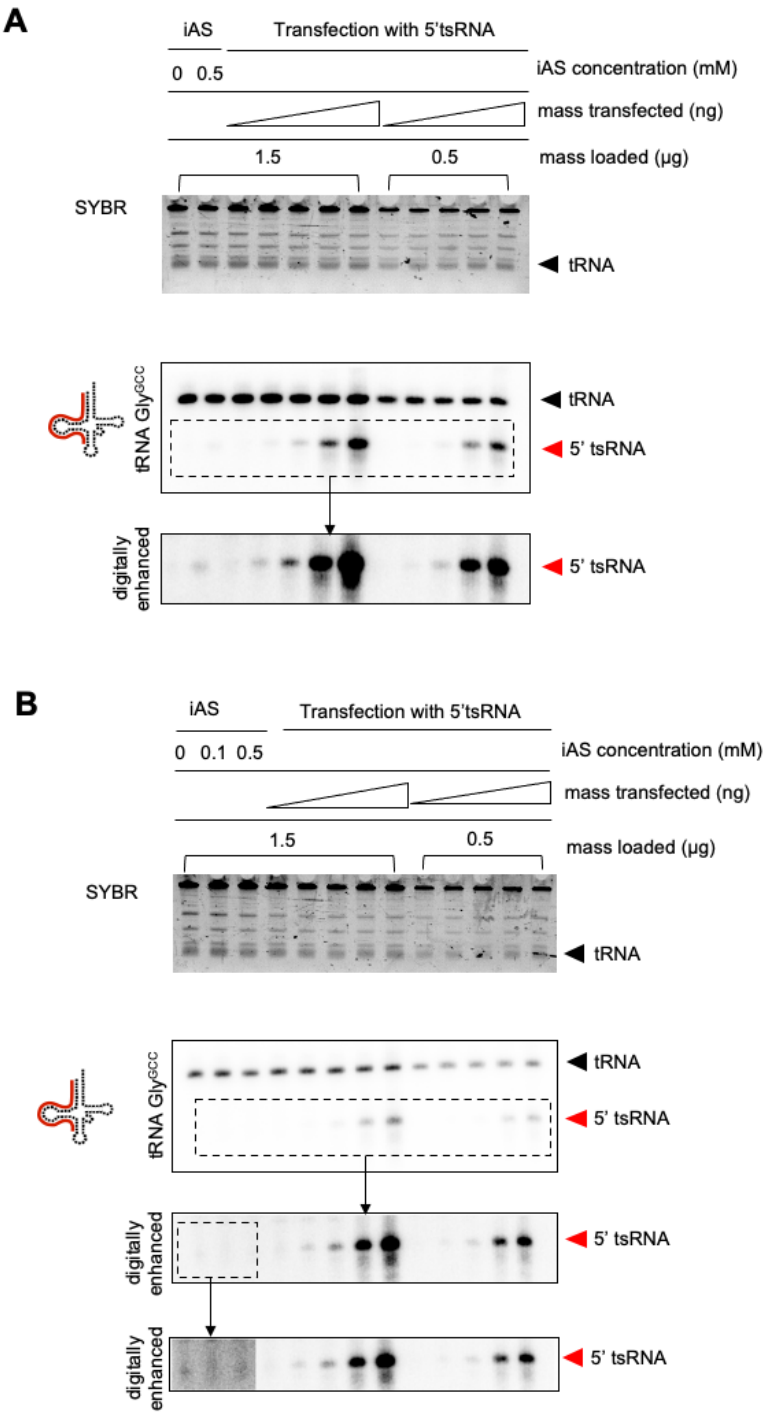


Figure 33. **tRNA fragmentation induced by 0.5 mM acute iAs treatment is comparable to transfection with 15 ng of synthetic 5' tsRNA.**

(A) 0.6 x 10^6 HeLa cells were treated with 0.5 mM iAs for one hour. 1.2 x 10^6 HeLa cells were transfected with 1.5, 15, 150, 1500 or 2500 ng of synthetic 5' tsRNA-Gly^{GCC} for 24 hours using lipofectamine. RNA was extracted and 1.5 or 0.5 µg of RNA separated on a 12% denaturing urea-PAGE and SYBR gold stained. **(B)** HeLa cells were treated with 0.1 and 0.5 mM $NaAsO_2$ for one hour and recovered for 24 hours in normal media or transfected with 1.5, 15, 150, 1500 or 2500 ng of synthetic 5' tsRNA-Gly^{GCC} for 24 hours using lipofectamine. RNA was extracted and 1.5 or 0.5 µg of RNA separated on a 12% denaturing urea-PAGE and SYBR gold stained.

5.3.7 Testing angiogenin for induction of tRNA fragmentation

Angiogenin is an endonuclease that hydrolyzes tRNAs specifically in the anti-codon loop, resulting in tRNA fragments. To approach the detection of endogenously produced tsRNAs without preceding transfection of synthetic 5' tsRNAs, HeLa cells treated with human recombinant angiogenin (hrANG), which can enter cells through various receptors (Hu et al. 1997). Experimental parameters such as duration of hrANG treatment as well as duration of HCR amplification were varied to assess the limits of signal detection from endogenously produced 5' tsRNAs.

First, hrANG treatment of cells was tested for two hours followed by a 24-hour recovery after hrANG treatment with different durations of HCR-based signal amplification (Figure 34).

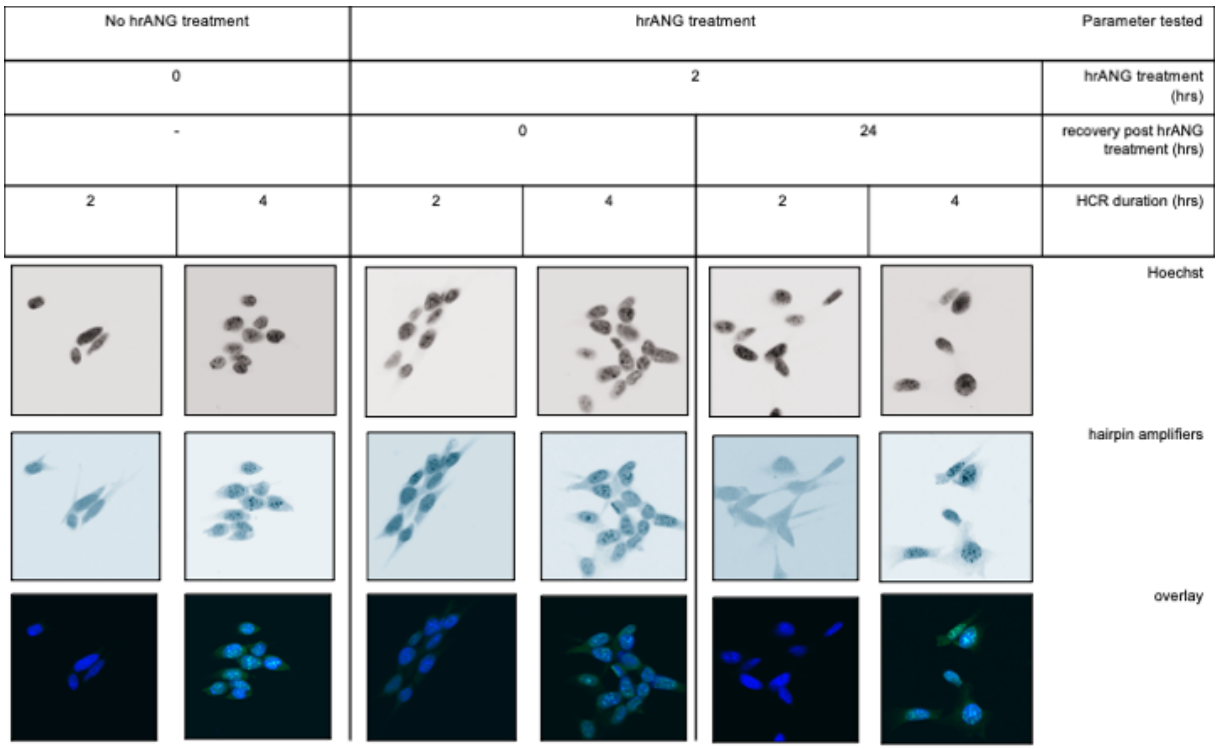


Figure 34. **hrANG treatment does not result in increased HCR signal.**

HeLa cells were grown on Geltrex-coated glass coverslips until confluent, then treated with 500 ng human recombinant angiogenin for two hours and optionally recovered for 24 hours in normal media, afterwards fixed with 4 % PFA and permeabilized with 0.1 µg/ml Proteinase K and incubated with 100 pmol of a compLINK over-night. Upon hybridization the coverslips were incubated over-night with 35 µM RtcB ligase to covalently link 5'tsRNA and compLINK. For stripping of background signal the coverslips were placed into 70 % formamide + 5x SSC at 70 °C for 30 minutes and placed into ice-cold 75 % EtOH afterwards. Subsequently, coverslips were incubated with 100 pmol of an HCR initiating oligonucleotide over-night. Hairpin assembly for HCR was carried out with 24 pmol of each hairpin amplifier for two and four hours. Analysis was performed by confocal microscopy.

The results showed that the HCR amplification signal was not affected by hrANG treatment. Importantly, the majority of the compLINK signal (Figure 2B) was localized to nuclear speckles likely representing nucleoli, which indicated signal creation at the level of tRNA-Gly^{GCC}. Recovery from hrANG treatment

also did not result in an increase of the HCR amplification signal. An increase of HCR signal was only observed with increasing the duration of the HCR amplification cycle.

To determine if the hrANG that was used in these *in vivo* experiments showed catalytic activity, RNAs were extracted from cells treated with hrANG and tRNA fragmentation was assessed by northern blotting using complementary probes against the 5' part of tRNA-Gly^{GCC}.

Notably, an increase of 5' tsRNAs could only be detected in one of the two replicates of hrANG-treated cells (Figure 36A). Compared to the serial dilution of synthetic 5' tsRNAs that were loaded onto the same gel, the intensity of hrANG-induced signals suggested that hrANG treatment resulted in between 50 and 100 pg 5' tsRNA-Gly^{GCC} per 1.5 μg total RNA that was extracted from approximately 0.6 x 10^6 cells.

Comparison between hrANG treatment and iAs treatment showed that hrANG treatment did not result in the production of 5' tsRNA-Gly^{GCC}, whereas iAs treatment resulted in a significant increase of 5' tsRNA-Gly^{GCC} (Figure 36B).

In conclusion, these results indicated that using hrANG on cultured cells to induce endogenous tRNA fragmentation was unsuccessful, which indicated the need for applying other methods for the induction of tRNA fragmentation in cells.

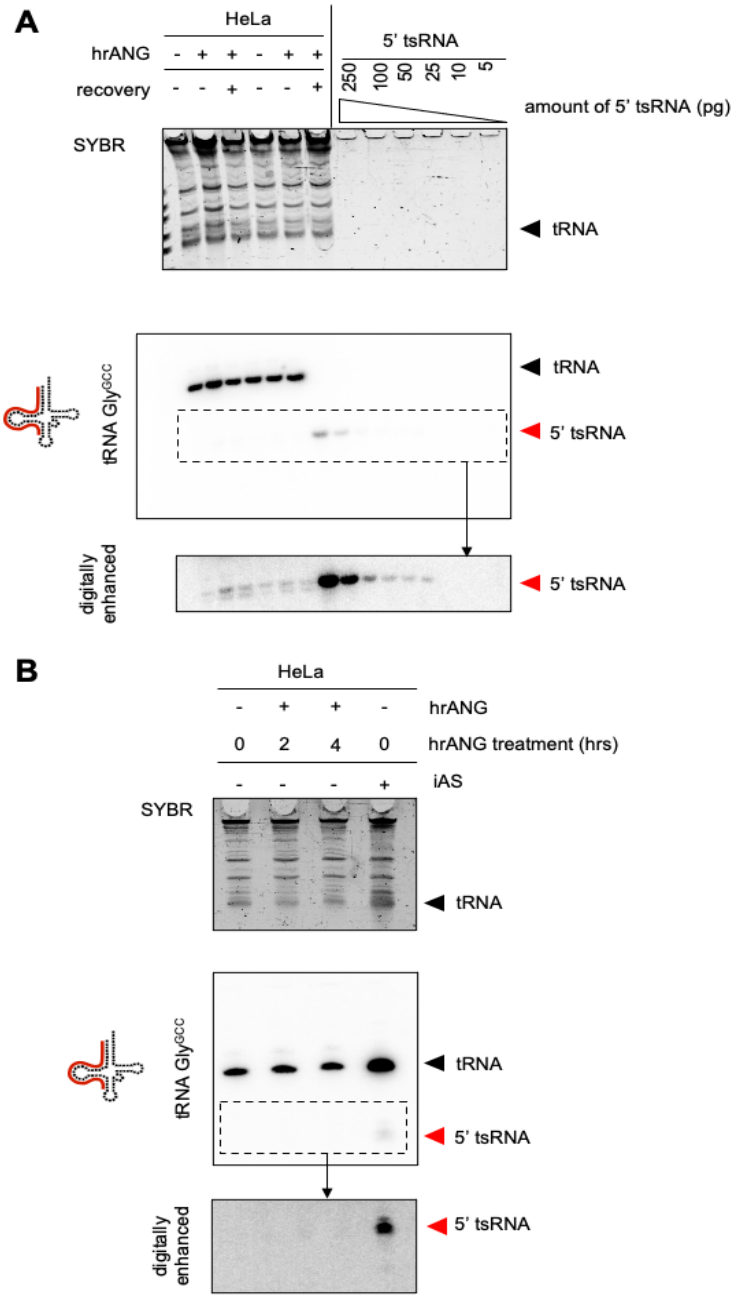


Figure 35. **Incubation of cells with hrANG fails to induce tRNA fragmentation.**

(A) HeLa cells were treated with 500 ng hrANG for two hours and selectively recovered for 24 hours in normal media in duplicate. RNA was extracted and 1.5 µg of RNA separated on a 12% denaturing urea-PAGE next to a serial dilution of synthetic 5' tsRNA-Gly^{GCC} and SYBR gold stained. Concentration of 5' tsRNA-Gly^{GCC} derived from HeLa cells was further collated with the concentration of synthetic 5' tsRNA by northern blotting against 5' tRNA Gly^{GCC}. **(B)** HeLa cells were treated with 500 ng hrANG for two and four hours or with 0.5 mM iAs for one hour and recovered for 24 hours in normal media. RNA was extracted and 1.5 µg of RNA separated on a 12% denaturing urea-PAGE followed by SYBR gold staining. tRNA fragmentation was detected by northern blotting for tRNA Gly^{GCC}.

6 Discussion

Cleavage of tRNAs by specific endonucleases upon stress generates tsRNAs. To investigate their subcellular localization a method for visualization by confocal microscopy was developed.

tsRNA *in vivo* visualization was based on the chemical properties of 5' tsRNA ends. To this end, the identity of 3' ends of tsRNAs was first corroborated *in vitro*. Thereupon, an approach for sequence-specific ligation of tsRNAs to complementary linker oligonucleotides (compLINKs) that would enable signalling *in vivo* was developed. Guided by *in vitro* results, signalling capacities of different signal amplification approaches were tested and compared to standard FISH methods.

The following discussion of this thesis is divided into two parts: the technical and the biological discussion.

6.1 Technical Discussion

6.1.1 Removal of cyclic phosphate

For the purpose of identifying a possible distinctive feature between tRNAs and tsRNAs the presence of 3' cyclic phosphate (cycP) upon stress induced cleavage was investigated. T4 PNK treatment was used to remove cycP of synthetic 5' tsRNA as well as of endogenously produced HEK cell-derived 5' tsRNAs. As previously published, treatment with T4 PNK is the standard in cycP-RNA-seq and used as a step for the removal of cycP to enable capturing of cycP-terminal RNAs by sequencing (Honda et al. 2016). While cycP could be clearly identified in synthetic tsRNAs, surprisingly, a treatment with T4 PNK was insufficient to monitor cycP in stress-induced tsRNAs, despite testing different masses of cellular RNA. These results would suggest application of a different method for clear identification of the cycP in cell-derived tsRNAs.

6.1.2 RtcB ligation in vitro

In vitro tests of RtcB ligase activity between synthetic 5' tsRNAs and compLINKs showed that increasing compLINK concentrations did not lead to improved ligation efficiency. compLINK concentrations exceeding the tsRNA concentration by a factor of ten even showed significantly less ligation between compLINK and tsRNA. These results suggest that the ligation efficiency decreased due to an overshoot of substrate. One possible solution for this was an increased concentration of enzyme. However, these findings were included for planning of *in vivo* experimentation.

Furthermore, the results also indicated that RtcB activity was not limited to ligation between ssRNA and other RNAs, but also occured between ssRNA and ssDNAs. These results are consistent with previously observed activity of RtcB on DNA (Das et al. 2013).

6.1.3 Visualization of hairpin-compLINKs

Challenges in visualization of hairpin-compLINKs on urea-PAGE occurred during *in vitro* testing. The results showed that the identification of respective compLINKs was facilitated by adaptation of the post-electrophoresis staining reagent. These results are surprising, considering that SYBR gold and Gelred both stain nucleic acids in gels with the same binding mechanism.

Another observation in hairpin-LINK tests in urea-PAGE was the formation of polymers of higher molecular weight that were visible as upshifted bands. Their appearance might be explained by self-assembly. While re-folding treatments were performed to aim at dissociating interactions between single hairpin-compLINKs, during the unfolded stage hybridization of specific sequence parts of the compLINKs could have occurred. That might also be the reason for high band intensities of polymers upon Gelred and SYBR gold staining after urea-PAGE. Both staining reagents can intercalate into double stranded structures, which might have formed due to hairpin compLINK self-assembly, resulting in strong staining of the polymer products.

6.1.4 *In vivo* transfection of 5’ tsRNAs

It was observed that the use of DOTAP and Lipofectamine for transfection both led to dot-like accumulations of fluorescently labelled synthetic 5’ tsRNAs mainly around the nucleus at the cytosolic phase. Transfection with Lipofectamine resulted in higher yield of fluorescent signal where tsRNAs accumulated compared to signal that was obtained from DOTAP transfection. Higher transfection efficiency by Lipofectamine has also been reported in previous literature (Wang et al. 2012), interestingly, transfection by Lipofectamine also led to less unspecific background signal. This could be explained by the concentration of fluorescent 5’ tsRNAs within liposomes during intracellular trafficking.

Tracking the transfected tsRNA several days after transfection with Lipofectamine revealed that 5’ tsRNAs were barely detectable from the fifth day post transfection, which suggests that transfected tsRNAs were degraded or dots of accumulated tsRNAs dispersed after a certain time and were not passed on during mitosis.

6.1.5 Conventional Fluorescence *in situ* Hybridization

Hybridization of fluorescently labeled compLINKs to transfected 5’ tsRNA was detectable by co-localization of Alexa488- and Atto590-signal, resulting in a yellow signal. Fluorescent green signal

deviating from co-localization signals indicated that compLINKs also hybridized to mature tRNAs. After application of the *in vitro* developed RtcB ligation approach und subsequent stripping the remaining signal of fluorescent compLINK was not sufficient to localize smaller amounts of tsRNAs. The observed signals indicate that the conventional FISH approaches are insufficiently specific, and the resulting signal yield would not enable localizing endogenous tsRNAs *in vivo*.

6.1.6 Hybridization-Chain-Reaction

Compared to conventional FISH the application of HCR showed a significant improvement in terms of signal production. Even the shortest duration of HCR cycle showed a strong signal amplification in the area of accumulated transfected 5' tsRNAs. However, it was observed that amplified signal also occurred in areas where no accumulation of the 5' tsRNAs occurred. This suggests that amplified signal originates from initiator sequences, that were not hybridized to the landing site of the first compLINK and were insufficiently removed by washing after the hybridization step. Another explanation might be that even after high stringency stripping a fraction of the first compLINK containing the landing site was hybridized to tRNAs and subsequently caused false signal.

Furthermore, comparison between the different durations of HCR cycles revealed that there are only minor differences in signal resulting from 4-hour versus 18-hour HCR cycles. Even when providing the reaction with proportional amounts of hairpin amplifiers to duration, the signal did not increase exponentially. This suggests that the DNA duplex formed by hairpin initiators might disassemble after a certain length of polymer is reached. It would be interesting to investigate limiting factors of the chain reaction behind hairpin assembly and to learn more about maximal signal yield and the optimization of reaction parameters. For that, the kinetics behind HCR would need further investigation.

6.1.7 Hybridization-Chain-Reaction 3.0

The signal yield from HCR 3.0 was significantly lower than signal obtained from HCR, with the difference that HCR 3.0 showed more specificity for the transfected target. The assumption that making signal amplification dependent on correct hybridization of the split initiator halves increased sensitivity was compatible with the observed loss of background signal. Interestingly, the use of shorter hairpin amplifiers resulted in less signal than use of longer hairpin amplifier designs, which is unexpected considering that assembly of longer hairpins bares a risk of misfolding and interruption of the chain reaction. In addition to that, the shorter hairpin design was identical to the hairpin amplifiers that were previously used in HCR, where hairpin assembly caused HCR signal. To that end, it would be interesting to further test different lengths of initiator platforms formed by initiator halves, as well as different lengths of hairpin amplifiers for increasing signalling efficiency.

6.2 Biological Discussion

The low abundance of endogenous tsRNAs that occur in cells after stress was one of the main limitations during development of the approach. Considering that, the development was based on transfection of tsRNAs. By signal production based on artificially increased numbers of target molecules, the minimal detectable amount needs to be taken into account before transferring findings to endogenously produced tsRNAs.

6.2.1 Induction of tRNA fragmentation with arsenite

Comparison of the amounts of tsRNA-Gly^{GCC} originating from acute stress treatment by inorganic arsenite (iAs) to the dilution titration of synthetic tsRNA, indicated that the amount detected for 0.5 M acute iAs stress is comparable to tsRNAs extracted from cells that were previously transfected with 15 to 150 ng of synthetic tsRNAs. Considering that the RNA from the transfection experiment was extracted from double the number of cells, and that the complementary *in vivo* transfection titration experiment still showed transfection signal that was amplified in parts, one could hypothesize that those small amounts of tsRNA are still sufficient for detection. As a prerequisite for detection of endogenous tsRNAs, the developed approach would need further optimization. HCR 3.0, for instance, would be promising method for detection of those lowly abundant small non-coding RNAs, because it showed barely any unspecific background signal.

The RNA extracted from cells that were recovered upon iAs treatment showed that 5’ tsRNA-Gly^{GCC} was detectable even 24 hours after stress, however, the levels were only traceable upon strong digital enhancement and no difference between 0.1 M and 0.5 M iAs could be detected.

Another limitation that occurred during iAs experimentation was detachment of cells from coverslips. Apart from cell debris, that was observed simultaneously, many cells were lost after treatment with arsenite, which is why this thesis does not provide data for *in vivo* signal detection after oxidative stress exposure. One possible solution for that problem would be testing different coating matrix solutions or testing the developed method in a different stress-paradigm.

6.2.2 Testing angiogenin for induction of tRNA fragmentation

HCR on human recombinant angiogenin (hrANG)-treated cells revealed signal amplification of nuclear speckles likely representing nucleoli, which indicated signal amplification at the level of tRNA-Gly^{GCC}. This signal was not distinguishable from possible signal originating from 5’ tsRNAs resulting from angiogenin-mediated cleavage of tRNAs. Therefore, the previously used hrANG was repeatedly tested followed by northern blotting for 5’ tRNA-Gly^{GCC}. The results showed 5’ tsRNAs only in one of the two

replicates, which was not considered significant. In another test, the same hrANG was used on cells and tRNA fragmentation was compared to fragmentation induced by treatment with 0.5 M iAs and recovery for 24 hours. The results indicated no induction of tRNA fragmentation mediated by hrANG, due to 0-, 2- and 4-hour treatment did not result in detectable amounts of tsRNAs, while iAs-mediated fragmentation could be clearly observed.

In summary, these results suggest that the used hrANG showed no catalytic activity and the signal amplification observed *in vivo* was only a result of background amplification. Despite absence of catalytic activity and therefore tsRNAs, considering the amount of background signal under inclusion of stripping confirms that detection of small amounts of tsRNAs by HCR could be problematic. Furthermore, it would be important to repeat the experiments with functional hrANG under incorporation of a control, that was not treated with hrANG and application of HCR 3.0, with the aim of reducing background signal.

6.3 Summary and Outlook

In summary, the data presented in this thesis shows that the specific chemical end-identity of 5' tsRNAs resulting from stress-induced cleavage can be used for differential detection and distinction from tRNAs. RtcB ligase can be used to covalently link tsRNAs to compLINKs which can further be utilized for tsRNA signal detection *in vivo*. The efficiency of ligation by RtcB can be assessed *in vitro* on denaturing urea-PAGE, allowing to test different compLINK designs.

Furthermore, the obtained results from the *in vivo* tests on different cell lines allow a valuation of different FISH-based methodologies, based on application suitability for the visualization of tsRNAs. Generally, signal amplification by HCR results in stronger signalling compared to conventional FISH methods, which makes it more suitable for detection of lowly abundant RNAs, as stress-induced 5' tsRNAs. Nonetheless, signals caused by compLINK hybridization to mature tRNAs lead to unspecific background signals, making it difficult to narrow down the signal effectively sourcing from tsRNAs. The specificity of the approach can demonstrably be increased by application of HCR 3.0, which not only reduces technical manipulation but also results in significantly less background signal.

HCR 3.0 is a useful approach to selectively amplify signals from tsRNAs. It revealed its potential to make non-transfected tsRNAs visualizable and also offers capacities for enabling multiplexing. In order to address these open points, HCR 3.0 should be further tested and a greater variety of compLINK designs should be evaluated to visualize endogenous tsRNAs of different identities upon stress.

Moreover, HCR and especially HCR 3.0 provide the opportunity of detecting tsRNAs in whole-mount tissues. For that, it would be important to test different parameters of the protocol and adapt compLINK designs to enable visualization of tsRNAs in whole-mount tissues of different origin.

7 Abstract

Transfer RNAs (tRNAs) are widely known as essential components of the translational machinery, acting as adaptor molecules during mRNA decoding. Apart from their function in translation, additional (non-canonical) roles have been linked to tRNAs.

One source of their complexity are tRNA-derived small RNAs (tsRNAs) which are small non-coding RNAs generated by enzymatic cleavage of tRNAs upon various conditions, including stress. tsRNAs have been implicated in a wide range of biological processes. However, apart from these conceptual insights, exact sub-cellular localization of tsRNAs still remains unclear, largely due to the lack of methodology allowing to distinguish tsRNAs from tRNAs.

This thesis is based on development of a protocol for the visualization of specific tsRNA in cells by confocal microscopy, which would allow contributing important information when studying the biological impact of this class of small RNAs.

8 Zusammenfassung

Transfer RNAs (tRNAs) sind bekannt als wichtige Bestandteile des Translationsprozesses, in dem sie als Adaptermoleküle für die Übersetzung von mRNA-Sequenzen fungieren. Neben ihrer Rolle in der Translation, können tRNAs jedoch auch zusätzliche Funktionen ausüben.

Ein Beispiel für diese Komplexität sind tRNA-abgeleitete kleine RNAs (tsRNAs). Diese kleinen nicht-kodierenden RNAs entstehen durch enzymatische Spaltung, die durch verschiedene Bedingung, wie zum Beispiel Stress, hervorgerufen werden kann. tsRNAs sind nachweislich an einer Vielzahl biologischer Prozesse beteiligt. Abgesehen von diesen konzeptionellen Erkenntnissen ist die genaue subzelluläre Lokalisation von tsRNAs allerdings weiterhin unbekannt. Dies ist hauptsächlich darauf zurückzuführen, dass es bisher keine Methode gibt, die es ermöglicht zwischen tsRNAs und tRNAs zu differenzieren.

Diese Arbeit basiert auf der Entwicklung eines Protokolls zur Visualisierung spezifischer tsRNAs in Zellen durch konfokale Mikroskopie. Dessen Etablierung kann wichtige Informationen zur Beantwortung von Fragen bezüglich der biologischen Bedeutung dieser Klasse von kleinen RNAs beitragen.

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12 Supplementary Information

12.1 Protocol for conventional FISH in adherent cells

Preparation

1) Coat sterilized glass coverslips with coating matrix (Geltrex, Vitronectin, Laminin).
2) Seed cells on pre-coated, pre-warmed slides in a 6-well plate and incubate at 37 °C over-night.
3) Induce tRNA fragmentation (iAs, hrANG) or transfect tRNA fragments (Lipofectamine, DOTAP)

Fixation and Permeabilization

4) Wash in 1x PBS for 10 minutes.
5) Fixation: remove PBS and fix in 4% PFA in PBS for 10 minutes.
6) Wash in 1x PBS for 10 minutes.
7) Permeabilize with 0.1 ug/mL Proteinase K in PK buffer for 10 minutes.
8) Wash in 1x PBS for 10 minutes.
9) Post-fixation: fix in 4% PFA in PBS for 15 minutes
10) Wash in 1x PBS for 10 minutes
11) Rinse in RNase-free H_2O
12) Acetylation with 0.25 % acetic anhydride in 0.1M triethanolamine (Acetylation solution) twice, 5 minutes each.
13) Wash in 1x PBS for 10 minutes. Optionally: store slides parafilm-sealed at 4 °C in 1x PBS or citrate buffer until start of protocol (up to one week).

Hybridization of fluorescent probe

14) Pre-hybridize coverslips in 15% FA hybridization solution without probe at 37 °C for 30 to 60 minutes (no shaking).
15) Incubate coverslips with fluorescently labelled complementary probe over-night at 37 °C in a humidifying chamber (100 pmol of Linker in 15% FA hybridization solution up to 200 µL per slide).

16) Next day: rinse coverslips with pre-warmed (37 °C) 2x SSCT.
17) Wash 3x in 2x SSCT at 37 °C, 10 minutes each.
18) Wash 2x in 5x SSCT at room temperature, 10 minutes each.

RtcB ligation

19) Rinse coverslips in SSC-washout solution.
20) Wash 2x in SSC-washout solution at RT, 5 minutes each.
21) Ligation preparation: Prepare the pre-ligation mix and add 100 µL of mix on top of each coverslip. Incubate at room temperature for 30 minutes in a humidifying chamber.
22) Prepare the RtcB ligation mix and add 200 µL of the mix as a droplet on a parafilm-covered surface in a humidifying chamber.
23) Remove the pre-ligation mix from the prepared coverslips and transfer the coverslip onto the RtcB mix droplet. Let it drop gently with the cells facing upside down towards RtcB mix.
24) Incubate the humidifying chamber at 37 °C over-night.

25) Next day: Wash 3x in 1x PBS at room temperature, 5’ each.
26) Wash in 2x SSC for 10 minutes.

Stripping

27) Place coverslips into 70% formamide + 5x SSC for 30 minutes at 70 °C.
28) Transfer coverslips into pre-chilled (-20 °C) 75% EtOH for 5 minutes.
29) Re-hydration by washing 3x in 1x PBS, 5 minutes each.

30) Stain in 1:10.000 Hoechst in 1x PBS for 10 minutes.
31) Wash in 1x PBS for 10 minutes.
32) Dip coverslip into distilled water and remove excess water by tapping gently on a paper towel.
33) Mount coverslip on top of a drop of mounting media.

12.2 Protocol for HCR in adherent cells

Preparation

34) Coat sterilized glass coverslips with coating matrix (Geltrex, Vitronectin, Laminin).
35) Seed cells on pre-coated, pre-warmed slides in a 6-well plate and incubate at 37 °C over-night.
36) Induce tRNA fragmentation (iAs, hrANG) or transfect tRNA fragments (Lipofectamine, DOTAP)

Fixation and Permeabilization

37) Wash in 1x PBS for 10 minutes.
38) Fixation: remove PBS and fix in 4% PFA in PBS for 10 minutes.
39) Wash in 1x PBS for 10 minutes.
40) Permeabilize with 0.1 ug/mL Proteinase K in PK buffer for 10 minutes.
41) Wash in 1x PBS for 10 minutes.
42) Post-fixation: fix in 4% PFA in PBS for 15 minutes
43) Wash in 1x PBS for 10 minutes
44) Rinse in RNase-free H_2O
45) Acetylation with 0.25 % acetic anhydride in 0.1M triethanolamine (Acetylation solution) twice, 5 minutes each.
46) Wash in 1x PBS for 10 minutes. Optionally: store slides parafilm-sealed at 4 °C in 1x PBS or citrate buffer until start of protocol (up to one week).

First Hybridization (Complementary probe)

47) Pre-hybridize coverslips in 15% FA hybridization solution without linker oligonucleotide at 37 °C for 30 to 60 minutes (no shaking).
48) Incubate coverslips with complementary linker oligonucleotide over-night at 37 °C in a humidifying chamber (100 pmol of probe in 15% FA hybridization solution up to 200 µL per slide).

49) Next day: rinse coverslips with pre-warmed (37 °C) 2x SSCT.
50) Wash 3x in 2x SSCT at 37 °C, 10 minutes each.
51) Wash 2x in 5x SSCT at room temperature, 10 minutes each.

RtcB ligation

52) Rinse coverslips in SSC-washout solution.
53) Wash 2x in SSC-washout solution at RT, 5 minutes each.
54) Ligation preparation: Prepare the pre-ligation mix and add 100 µL of mix on top of each coverslip. Incubate at room temperature for 30 minutes in a humidifying chamber.

55) Prepare the RtcB ligation mix and add 200 µL of the mix as a droplet on a parafilm-covered surface in a humidifying chamber.
56) Remove the pre-ligation mix from the prepared coverslips and transfer the coverslip onto the RtcB mix droplet. Let it drop gently with the cells facing upside down towards RtcB mix.
57) Incubate the humidifying chamber at 37 °C over-night.

58) Next day: Wash 3x in 1x PBS at room temperature, 5' each.
59) Wash in 2x SSC for 10 minutes.

Stripping

60) Place coverslips into 70% formamide + 5x SSC for 30 minutes at 70 °C.
61) Transfer coverslips into pre-chilled (-20 °C) 75% EtOH for 5 minutes.
62) Re-hydration by washing 3x in 1x PBS, 5 minutes each.

Second hybridization (initiator sequence)

63) Pre-hybridize coverslips in 15% FA hybridization solution without oligonucleotide at 37 °C for 30 to 60 minutes (no shaking).
64) Incubate coverslips with initiator sequence-containing oligonucleotide over-night at 37 °C in a humidifying chamber (100 pmol of Linker in 15% FA hybridization solution up to 200 µL per slide).

65) Next day: rinse coverslips with pre-warmed (37 °C) 2x SSCT.
66) Wash 3x in 2x SSCT at 37 °C, 10 minutes each.

HCR amplification

67) Pre-amplification for 30 minutes at room temperature in amplification buffer.
68) Prepare a hairpin working solution with 24 pmol of every hairpin amplifier in 400 µL amplification buffer (final concentration: 24 pmol hairpin amplifier/200 µL amplification buffer).
69) HCR: add mix onto slides and incubate 4 hrs in the dark at room temperature.

70) Rinse off excess hairpins with 5x SSCT.
71) Wash 3x in 5x SSCT, 10 minutes each.
72) Wash 3 x in 1x PBS, 5 minutes each.
73) Stain in 1:10.000 Hoechst in 1x PBS for 10 minutes.
74) Wash in 1x PBS for 10 minutes.
75) Dip coverslip into distilled water and remove excess water by tapping gently on a paper towel.
76) Mount coverslip on top of a drop of mounting media.

12.3 Protocol for HCR 3.0 in adherent cells

Preparation

1) Coat sterilized glass coverslips with coating matrix (Geltrex, Vitronectin, Laminin).
2) Seed cells on pre-coated, pre-warmed slides in a 6-well plate and incubate at 37 °C over-night.
3) Induce tRNA fragmentation (iAs, hrANG) or transfect tRNA fragments (Lipofectamine, DOTAP)

Fixation and Permeabilization

4) Wash in 1x PBS for 10 minutes.

5) Fixation: remove PBS and fix in 4% PFA in PBS for 10 minutes.
6) Wash in 1x PBS for 10 minutes.
7) Permeabilize with 0.1 ug/mL Proteinase K in PK buffer for 10 minutes.
8) Wash in 1x PBS for 10 minutes.
9) Post-fixation: fix in 4% PFA in PBS for 15 minutes
10) Wash in 1x PBS for 10 minutes
11) Rinse in RNase-free H_2O
12) Acetylation with 0.25 % acetic anhydride in 0.1M triethanolamine (Acetylation solution) twice, 5 minutes each.
13) Wash in 1x PBS for 10 minutes. Optionally: store slides parafilm-sealed at 4 °C in 1x PBS or citrate buffer until start of protocol (up to one week).

First Hybridization (hairpin compLINK)

14) Pre-hybridize coverslips in hybridization solution without formamide without the hairpin-compLINK at 37 °C for 30 to 60 minutes (no shaking).
15) Incubate coverslips with hairpin-compLINK over-night at 37 °C in a humidifying chamber (100 pmol of compLINK in hybridization solution without formamide up to 200 µL per slide).

16) Next day: rinse coverslips with pre-warmed (37 °C) 2x SSCT.
17) Wash 3x in 2x SSCT at 37 °C, 10 minutes each.
18) Wash 2x in 5x SSCT at room temperature, 10 minutes each.

RtcB ligation

19) Rinse coverslips in SSC-washout solution.
20) Wash 2x in SSC-washout solution at RT, 5 minutes each.
21) Ligation preparation: Prepare the pre-ligation mix and add 100 µL of mix on top of each coverslip. Incubate at room temperature for 30 minutes in a humidifying chamber.
22) Prepare the RtcB ligation mix and add 200 µL of the mix as a droplet on a parafilm-covered surface in a humidifying chamber.
23) Remove the pre-ligation mix from the prepared coverslips and transfer the coverslip onto the RtcB mix droplet. Let it drop gently with the cells facing upside down towards RtcB mix.
24) Incubate the humidifying chamber at 37 °C over-night.

25) Next day: Wash 3x in 1x PBS at room temperature, 5’ each.
26) Wash in 2x SSC for 10 minutes.

Second hybridization (split initiators)

27) Pre-hybridize coverslips in 30% formamide hybridization solution without split initiators at 37 °C for 30 to 60 minutes (no shaking).
28) Incubate coverslips with both split initiator oligonucleotides over-night at 37 °C in a humidifying chamber (100 pmol of split initiator in 15% FA hybridization solution up to 200 µL per slide).

29) Next day: wash coverslips with pre-warmed (37 °C) 30% formamide probe wash buffer.
30) Wash 2x in 5x SSCT at 37 °C, 10 minutes each.

HCR amplification

31) Pre-amplification for 30 minutes at room temperature in amplification buffer.
32) Prepare a hairpin working solution with 18 pmol of every hairpin amplifier in 300 µL amplification buffer (final concentration: 18 pmol hairpin amplifier/150 µL amplification buffer).
33) HCR: add mix onto slides and incubate over-night in the dark at room temperature.

34) Rinse off excess hairpins with 5x SSCT.
35) Wash 5x in 5x SSCT, 5 minutes each.

36) Wash 3 x in 1x PBS, 5 minutes each.
37) Stain in 1:10.000 Hoechst in 1x PBS for 10 minutes.
38) Wash in 1x PBS for 10 minutes.
39) Dip coverslip into distilled water and remove excess water by tapping gently on a paper towel.
40) Mount coverslip on top of a drop of mounting media.