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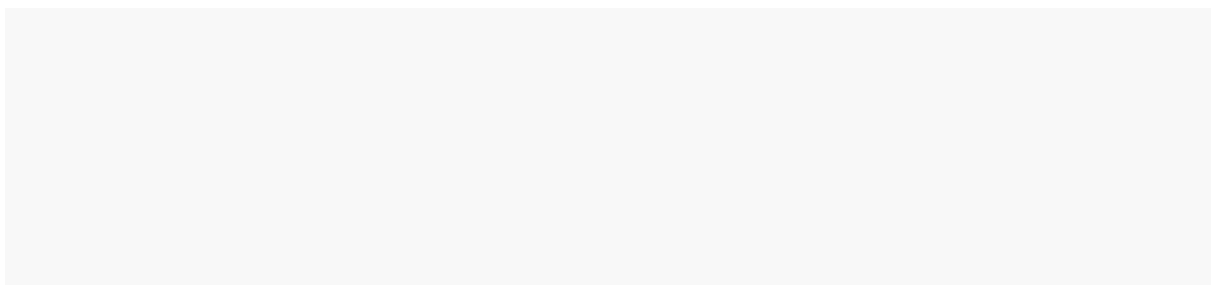
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1 Fluorescent labeling of plasmid DNA and messenger RNA: gains and losses of current labeling  
2 strategies

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## 7 **Abstract**

8 Live-cell imaging has provided the life sciences with insights into the cell biology and  
9 dynamics. Fluorescent labeling of target molecules proves to be indispensable in this regard. In  
10 this review, we focus on the current fluorescent labeling strategies for nucleic acids, and in  
11 particular messenger RNA (mRNA) and plasmid DNA (pDNA), which are of interest to a broad  
12 range of scientific fields. By giving a background of the available techniques and an evaluation  
13 of the pros and cons, we try to supply scientists with all the information needed to come to an  
14 informed choice of nucleic acid labeling strategy aimed at their particular needs.

15 Keywords: Fluorescent labeling methods, nucleic acids, mRNA, pDNA, live-cell imaging

## 16 **Introduction**

17 Ever since Robert Hooke and Anton van Leeuwenhoek used a single lens to visualize and  
18 describe simple biological samples in the 17<sup>th</sup> century, scientists relied on advances in  
19 technology to reveal more of nature's details. Image quality was improved in the following two  
20 centuries by optimized lens combinations and progress in glass production, but resolution,  
21 contrast, noise, optical aberrations, sensitivity and specificity were still major problems.  
22 Progression towards modern microscopy commenced when halfway the 19<sup>th</sup> century,  
23 fluorescence was being described by Stokes <sup>1</sup> and Von Lommel, creating the awareness that  
24 substances could be identified by measuring their specific fluorescence spectrum. Hence, one  
25 of the most importance principles of fluorescence microscopy was born. Around the same time,  
26 Ernst Abbe published his theoretical analysis of the role of diffraction in optical microscopy <sup>2</sup>.  
27 It was however only at the start of the 20<sup>th</sup> century that a prototype of a bright field fluorescence  
28 microscope was built at the Carl Zeiss factory. Continuous instrumentation development and  
29 fluorescent probe design was conducted over the next 50 years and with the invention of the  
30 beam splitting plate (dichroic mirror) <sup>3</sup>, the basis of the fluorescence microscope as we know it  
31 today was formed <sup>4, 5</sup>.

32 The use of fluorophores to color specific substances of interest, became indispensable in  
33 biology. While some molecules have intrinsic fluorescence properties, most biological  
34 substances have to be made visible by 'labeling' them with specific fluorophores. Once labeled,  
35 some molecules can be used as a probe to identify cellular structures, such as for example the  
36 use of labeled antibodies to detect protein location. Alternatively, the labeled molecules  
37 themselves are the subject of investigation and their intracellular distribution and the biological

1 processes in which they are involved are directly monitored. The choice for a certain  
2 fluorophore and an appropriate labeling strategy will greatly depend on the biological process  
3 one wishes to study. Ideally, the fluorophores specifically label the molecule of interest without  
4 non-specific binding to other intracellular structures. Also, the fluorophores should have a high  
5 brightness and limited photobleaching to allow for imaging of biological processes during a  
6 suitable time frame. Finally, the fluorophores should show no or only limited interference with  
7 the process one wishes to study, to avoid labeling-induced artifacts. There is a wide variety of  
8 fluorophores available with output wavelengths in the blue, green, yellow, orange, red, far-red  
9 or near-infrared region of the emission spectrum. Obviously, the choice of suitable output  
10 wavelengths is strongly dictated by the optics of the available fluorescence microscopes. Also  
11 the labeling strategy itself and the biological application (e.g. live-cell imaging versus fixed  
12 samples) will further determine the availability of compatible fluorophores. In Table 1, an  
13 overview of common fluorescent dye families is presented with available functionalities and  
14 photophysical properties.

15 One of the biological processes that is of interest in the field of non-viral nucleic acid delivery,  
16 is the hurdles nucleic acids face on their extracellular and intracellular journey. For decades,  
17 researchers have fluorescently labeled nucleic acids with a variety of labeling strategies to gain  
18 insight in the fate of these nucleic acids. While labeling short interference RNA (siRNA) or  
19 antisense oligonucleotides is primarily done during chemical synthesis by the manufacturer,  
20 several strategies exist for the labeling of larger polynucleotides, such as plasmid DNA (pDNA)  
21 and messenger RNA (mRNA), in a research lab. These polynucleotides are of interest for any  
22 biological application in which the expression of proteins is beneficial. The main obstacle to  
23 the wide-spread use of these pDNA and mRNA based therapies is the low efficiencies obtained  
24 with the current transfection methods. Negatively charged nucleic acids are mostly complexed  
25 with positively charged carriers to enhance cellular uptake of the complexes which are formed.  
26 Designing new delivery vehicles is crucial for the future success of these treatments. Live-cell  
27 imaging will undeniably play an important role in unraveling the different steps of the  
28 transfection pathway <sup>6</sup>.

29 This review focusses on methods to add fluorescent labels to polynucleotides, with the idea to  
30 image them in a biological environment. Labeling strategies which are compatible with live-  
31 cell imaging are of particular interest. Both methods that label polynucleotides prior to  
32 delivering them to living cells, as well as “in situ” labeling methods will be discussed.  
33 Polynucleotides have a well-defined primary and secondary structure, which enables yet  
34 another classification of fluorophore interaction which is based on their sequence specificity.  
35 Fluorophores that exhibit no sequence specificity interact with polynucleotides in a random  
36 fashion and are spread all over the polynucleotide chain. Sequence-specific polynucleotide  
37 labeling, on the other hand, only occurs at a predefined primary sequence, enabling control over  
38 the exact location of the labels on the target polynucleotide. In this review, the current available  
39 labeling strategies for DNA-based and RNA-based polynucleotides are classified based on their  
40 ability to label the nucleic acids in a random or sequence-specific way, with a special focus on  
41 the pros and cons of each labeling method that is being discussed.

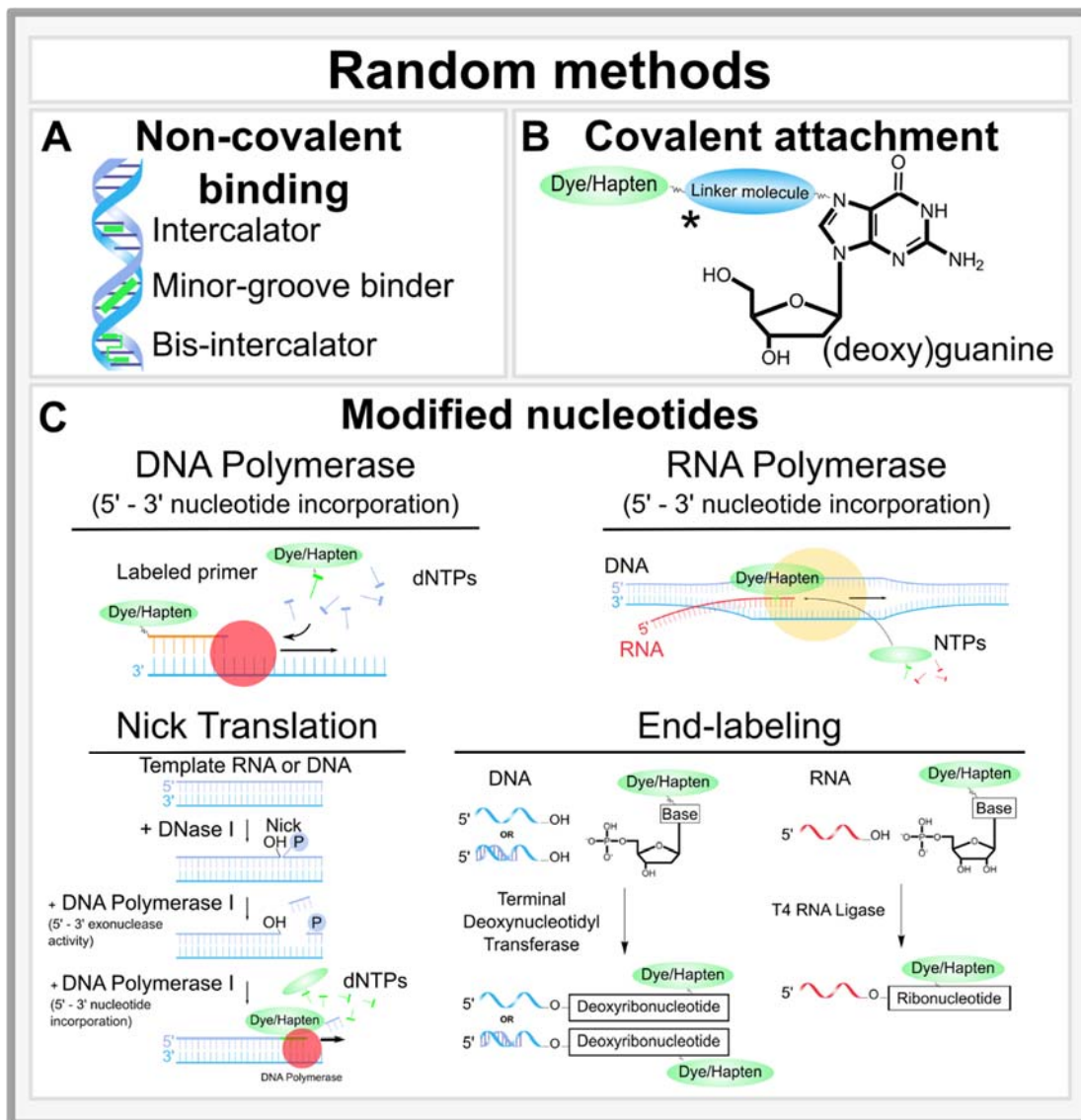
1 Table 1 Overview of fluorescent dye classes often used in nucleic acid labeling

Fluorescent dye class	Functionalities	Photophysical properties				Supplier	
		$\lambda_{\text{Abs.}}$ (nm)	$\lambda_{\text{Em.}}$ (nm)	Quantum yield	Remarks		
<b>Cyanine (Cy<sup>®</sup>) dyes</b>	Cy <sup>®</sup> 3	SE, CA, strep, mal, azide, alkyne,	550	570	0.15	Broad range of applications, less bright than Alexa Fluor <sup>®</sup> 546	Invitrogen, Jena Bioscience, GE Healthcare Life Sciences, Lumiprobe, ...
	Cy <sup>®</sup> 5	NH <sub>2</sub> , UTP, CTP, GTP, ATP, pCp, dUTP, dCTP	649	670	0.27	Broad range of applications, less bright than Alexa Fluor <sup>®</sup> 647	
<b>Cyanine dimers</b>	YOYO-1	N/A	491	509	0.38 <sup>†</sup> , 0.25 <sup>‡,7</sup>	Photocleavage of NA and bleaching reported <sup>8</sup>	Molecular Probes <sup>™</sup>
	TOTO-3		642	660	0.06	Bleaches fast (20-30s) <sup>9</sup>	
<b>Ethidium bromide</b>	N/A		285	605	0.20	Especially used for gel staining, but suspected mutagenicity	Invitrogen, Sigma-Aldrich, Bio-Rad, ...
<b>Alexa Fluor<sup>®</sup></b>	Alexa Fluor <sup>®</sup> 488	SE, CA, strep, mal, azide, alkyne,	495	519	0.92	Fluorescence output unmatched by any other spectrally similar dye	Molecular Probes <sup>®</sup>
	Alexa Fluor <sup>®</sup> 546	UTP, dUTP	556	573	0.79	Cy <sup>®</sup> 3 and TM-Rhodamine substitute	
	Alexa Fluor <sup>®</sup> 594	SE, CA, strep, mal, azide, alkyne, dUTP	590	617	0.66	Bright and photostable	
	Alexa Fluor <sup>®</sup> 647	SE, CA, strep, mal, azide, alkyne, ATP, dUTP, dCTP	650	668	0.33	brighter substitute for Cy <sup>®</sup> 5	
<b>ATTO dyes</b>	ATTO 488	SE, CA, strep, mal, azide, alkyne,	501	523	0.80	Very high photostability	ATTO-TEC, Sigma-Aldrich, Jena Bioscience
	ATTO 647N	NH <sub>2</sub> , biotin, UTP, ATP, dUTP, dCTP	644	669	0.65	Cy <sup>®</sup> 5 substitute, high thermal and photostability, exceptionally high stability towards atmospheric ozone	
<b>Fluorescein</b>	FITC (isothiocyanate)	SE, mal, azide, alkyne, UTP, CTP, ATP, dUTP, dCTP, dGTP, dATP	494	518	0.86 (0.1 N NaOH)	pH sensitive, prone to photobleaching	Thermo Scientific <sup>™</sup> , Sigma-Aldrich, Jena Bioscience, ...

	5-FAM (carboxylic acid)	SE, azide, alkyne, strep, NH <sub>2</sub> , dUTP, dCTP	494	518	0.90	SE form more stable bond with amines compared to FITC-SE	Molecular Probes™, Sigma-Aldrich, Jena Bioscience, Lumiprobe, ...
<b>TM- Rhodamine</b>	TRITC (isothiocyanate)	SE, strep, mal, azide, dUTP, dCTP	555	580	0.10	Prone to aggregation	Thermo Scientific™, Sigma-Aldrich, ...
	5-TAMRA (carboxylic acid)	SE, strep, mal, azide, alkyne, NH <sub>2</sub> , dUTP, dCTP	555	580	0.10	More stable than TRITC	Molecular Probes™, Sigma-Aldrich, AnaSpec.
<b>CX- Rhodamine</b>	5-ROX	SE, mal, azide, alkyne	576	591	0.70 (PO <sub>4</sub> buffer, pH 9)	Unstable compared to other rhodamines	Molecular Probes™, Sigma-Aldrich, Jena Bioscience, AnaSpec.
<b>Fluorescent proteins</b>	EGFP	N/A	488	509	0.60	Most used, brighter variants available, no problems with photostability	Thermo Scientific™, Sigma-Aldrich, BioVision
	mCherry	N/A	587	610	0.22	Superior photostability when compared to other red proteins	BioVision, Abcam®
<b>Quantum dots</b>	CdSe-ZnS	CA, strep, mal, azide, alkyne (cyclooctyne), NH <sub>2</sub> , biotin			0.20-0.50*	Bright, photostable, broad excitation, tunable narrow emission, but concerns about cell toxicity	Molecular Probes™, Sigma-Aldrich, Mesolight
<b>Lanthanide chelate</b>	Eu(III)-chelates	SE, CA, strep, mal, azide, alkyne,	376	616		Narrow emission peaks, long fluorescence lifetimes, high photostability but often needs antenna to transfer absorbed excitation energy to the lanthanide	PerkinElmer,
	Tb(III)-chelates	NH <sub>2</sub> , biotin	340	545	~1 (2H <sub>2</sub> O <sup>10</sup> )		Invitrogen, PerkinElmer
<b>Transient metal complexes</b>	Pt(II), Ir(III), Ru(II), Re(I)based	SE, CA, strep, mal, azide, alkyne, NH <sub>2</sub> , biotin, dUTP				Long fluorescence lifetimes interesting for FLIM, tunable excitation/emission, high photostability, good quantum yield	Sigma-Aldrich

SE: Succinimidyl ester, CA: carboxylic acid, strep: streptavidin, mal: maleimide, NH<sub>2</sub>: amine, †: double strand DNA, ‡: single strand DNA, \*: dependent on shell thickness<sup>11</sup>, FLIM: Fluorescence-Lifetime Imaging Microscopy

# 1 At random attachment of fluorophores to polynucleotides



2

3 **Figure 1 Overview of at random labeling methods. (A) Different modes of intercalation, (B)**  
 4 **Schematic overview of random covalent attachment, (C) Different techniques used to incorporate**  
 5 **modified nucleotides into DNA and RNA. dNTP: deoxynucleotide triphosphate, NTP: nucleotide**  
 6 **triphosphate. \*: Linker molecule structures can be found in Slattum (Mirus kit), Daniel et al.**  
 7 **(FastTag) and van Gijlswijk et al. (Universal Linkage System)**

8 In general, at random attachment requires little to no knowledge of the exact primary sequence  
 9 of the polynucleotide that needs to be labeled. Not surprisingly, it is being used by the oldest,  
 10 easiest and most commonly used labeling methods around. Random labeling of polynucleotides  
 11 can occur based on non-covalent or covalent interactions of the fluorophores with the  
 12 polynucleotides backbone. Additionally, the random incorporation of fluorescent nucleotides  
 13 during polynucleotide synthesis will be discussed.

14 Non-covalent nucleic-acid binding dyes

1 Two modes of random non-covalent interactions with nucleic acids are considered, namely  
2 groove binding and insertion of a fluorescent tag between base pairs, which is called  
3 intercalation (see Figure 1 A). The family of molecules with nucleic acid-binding properties is  
4 fairly large and diverse and binding mechanisms are often not (yet) defined<sup>15</sup>, but it is believed  
5 most of them exhibit only one of the two binding modes (e.g. YOYO-1<sup>16</sup>), but the combination  
6 of both has also been reported for PicoGreen<sup>®17</sup>.

7 Groove binding dyes, as the name indicates, interact with the major or the minor groove of a  
8 helix structure. In nucleic acids, these grooves are found in the  $\alpha$ -helix of double stranded DNA.  
9 Reactions in the major groove of DNA are more common for molecules reacting directly with  
10 the bases like for example the covalent labeling methods which will be discussed later, and  
11 proteins<sup>18</sup>. Minor-groove binding dyes are widely used and interact with the DNA in a non-  
12 covalent manner. These interactions are based on the shape of the minor groove, which should  
13 be accommodating for the dye (see Figure 1 A). A good match will maximize the stability of  
14 the van der Waal's contacts between target and dye. Next to these contacts, hydrogen bonds  
15 and electrostatic stabilizing interactions between the base pairs in the groove floor and the dye  
16 are the main contributors to a stable minor-groove binding. Additionally the increase in entropy  
17 due to the expulsion of bound water molecules from the minor groove will contribute slightly  
18 to the stability<sup>19</sup>. The effect these interactions have on the DNA morphology will differ from  
19 dye to dye, but in general an elongation of the total length and persistence length and DNA  
20 topology could be observed when using the minor-groove binding dye DAPI<sup>20</sup>.

21 Intercalation, on the other hand, is generally defined as the reversible insertion of molecules  
22 between layered structures. In polynucleotides, these layered structures consist of the stacked  
23 base pairs which form the secondary structure of DNA and RNA. Intercalating dyes are often  
24 cationic molecules with planar aromatic rings that insert between the base pairs of the helix-  
25 structure. During intercalation,  $\pi$ -stack interactions (intercalated moiety), hydrogen-bonding,  
26 van der Waals interactions, hydrophobic interactions and steric hindrance effects all play a  
27 role<sup>21</sup>. Most of them will exhibit a strong fluorescence enhancement upon binding. Intercalators  
28 can be found either as a monomer (intercalator) or combined in a dimer (bis-intercalation) for  
29 stronger binding and higher brightness (see Figure 1 A). Next to this advantage, monomers and  
30 dimers will also exhibit a different affinity for a given polynucleotide, such as single stranded  
31 vs. double stranded DNA or RNA, since intercalation is dependent on the secondary and tertiary  
32 structure of the nucleic acid. A good example is the monomer TOPRO-3 which does not stain  
33 RNA, while its dimer TOTO-3 does stain RNA<sup>9</sup>.

34 Like groove binding, the insertion of one (or two) dyes between the stacked bases will induce  
35 unwinding and lengthening of the nucleic acid helix structure and may subsequently disturb  
36 polynucleotide function. Hence, some intercalating dyes are potentially mutagenic by  
37 interfering with replication, transcription and DNA repair processes. The best known example  
38 is ethidium bromide, which is used for staining of agarose gels and subsequent nucleic acid  
39 quantitation. Ethidium Bromide is more and more being replaced by less toxic alternatives.  
40 Cyanine stains (like the TOTO and TOPRO family of dyes<sup>22</sup>)(see Table 1), for example, seem  
41 safer but often need cell permeabilization or other membrane disruptive techniques for cell  
42 staining. Martin et al. therefore compared several intercalating labeling methods for their use

1 in staining the nuclei during live-cell imaging (so-called counterstaining). The dyes were scored  
2 on cell permeability, live/fixed cell use, RNA staining and bleaching behavior. They came to  
3 the conclusion that the minor-groove binding dyes Hoechst 33258 and DRAQ5 are the most  
4 suitable stains for live-cell imaging, wherein the latter dye is reported to have more favorable  
5 optical properties. In the search for brighter fluorophores that exhibit better resistance against  
6 photobleaching, different metal complexes with intercalating or groove binding properties were  
7 considered. Among the elements from the lanthanide group, Europium (Eu) (III) and Terbium  
8 (Tb) (III) are two ions which possess interesting photophysical properties with a large Stokes'  
9 shift, long emission lifetimes, interesting for fluorescence-lifetime imaging, and high resistance  
10 to photobleaching (see Table 1)<sup>23</sup>. When these ions are complexed in a ligand structure, some  
11 complexes, like the conjugation of a naphthalene diimide derivative moiety with two  
12 luminescent chelates of tetradentate  $\beta$ -diketone-Ru<sup>3+</sup>, were used to specifically intercalate in  
13 double stranded DNA<sup>24</sup>. Similar properties can be found in the transition metal complexes with  
14 Pt(II) or d6 metal ion complexes (Ir(III), Ru(II) and Re(I) complexes)(see Table 1)<sup>25, 26</sup>. Square  
15 planar Pt(II) terpyridyl complexes were first reported to be DNA intercalators in the seventies<sup>27</sup>.  
16 More recently "Ru(II) bisimine complexes" were verified and used as DNA binders<sup>28</sup>. It should  
17 be noted that different isomeric forms of the same complex might result in different affinities.  
18  $\Delta$ -[Ru(phen)3]<sup>2+</sup> is for example a DNA intercalating complex while the  $\Lambda$  isomer prefers minor-  
19 groove binding<sup>25</sup>. Alternatively when one lanthanide complex and 2 platinum complexes are  
20 conjugated in a so-called heterometallic hairpin structure, bis-intercalation is seen<sup>29</sup>.

21 Next to the use of intercalating dyes for counterstaining of nuclei or staining of dead cells (e.g.  
22 propidium iodide)<sup>30</sup>, for which they are known since decades, intercalating dyes are good  
23 candidates for super resolution imaging of DNA<sup>31</sup>. The photochemical properties of the minor-  
24 groove binding dye Picogreen<sup>®</sup> makes it possible for direct Stochastic Optical Reconstruction  
25 Microscopy (dSTORM) to give insight into the dynamics of DNA organization after direct  
26 DNA labeling<sup>32</sup>.

27 Using intercalating dyes is probably the easiest method, since it solely requires mixing a dye  
28 with the target polynucleotide. Intercalating dyes are often used in quality control, nucleic acid  
29 quantification (e.g. quantitative polymerase chain reaction (PCR)) and as counterstain for the  
30 visualization of (live-) cell processes. Due to their interactions with the structure of the  
31 polynucleotides<sup>18, 20, 33, 34</sup>, influence on protein-nucleic acid interaction<sup>35</sup> and low brightness  
32 they are however not used extensively for labeling polynucleotides with the intention to follow  
33 their intracellular fate in living cells.

#### 34 Random covalent attachment of fluorophores and haptens

35 To prevent possible redistribution of intercalating dyes to non-target nucleic acids, covalently  
36 attaching fluorophores to the target nucleic acid is an interesting option. The irreversible  
37 binding of an organic dye or hapten to the nucleic acid will secure a stable and bright fluorescent  
38 signal. Haptens are defined as small molecules that can be detected by specific antibodies. After  
39 binding to nucleic acids, they allow subsequent immunodetection or affinity-bases purification.  
40 When biotin is used as hapten, any streptavidin or avidin coupled molecule can be coupled to  
41 the biotinylated nucleic acids through the strong and specific (strept)avidin-biotin interaction.



1 One of the most frequently encountered examples of covalent modification of exogenous  
2 polynucleotides is the use of the commercially available Label-IT Nucleic Acid Labeling Kit  
3 (Mirus bio LLC, Madison, WI, U.S.A.). Slattum demonstrated that reagents with an aromatic  
4 nitrogen mustard reactive center covalently alkylate nucleotides, primarily at the N<sup>7</sup> of  
5 (deoxy)guanine residues (see Figure 1 B). The available Label-IT reagents enable the direct  
6 coupling of fluorophores to the DNA structure (e.g. Cy3, Cy5, fluorescein, CX-rhodamine and  
7 TM-rhodamine, see Table 1), or the coupling of haptens like biotin that offer the possibility to  
8 couple any streptavidin or avidin conjugated molecule in a subsequent labeling reaction. It has  
9 been described that the transfection efficiency of pDNA labeled with the Label-IT kit is  
10 decreased when compared to unlabeled plasmids in a labeling density dependent manner <sup>12, 36</sup>.  
11 For pDNA transfections using liposomal carriers, endosomal escape, DNA dissociation from  
12 the carrier and transcription were reported to be affected, probably due to the hydrophobicity  
13 and steric hindrance of the fluorescent labels <sup>36</sup>. Also, Gasiorowski and Dean reported that  
14 nuclear retention of pDNA was affected by this labeling strategy. The labeling density of the  
15 nucleic acids can be controlled by titrating the amount of label-IT reagent for a given amount  
16 of nucleic acids. Ideally, the lowest labeling density that is still detectable by the used  
17 instrumentation should be preferred. Despite these caveats, this labeling method is useful,  
18 among other things, for intracellular tracking of polynucleotides to elucidate cellular  
19 mechanisms. Rhodamine labeled pDNA was for example imaged during cytoplasmic transport  
20 over the tubulin network after transfection <sup>38</sup>. The labeling of pDNA with Cy3<sup>®</sup> and Cy5<sup>®</sup>, on  
21 the other hand was used to elucidate the cellular internalization mechanisms after transfection  
22 with polyethyleneimine (PEI) <sup>39</sup>. Similarly, Cy5<sup>®</sup> pDNA was used to image the internalization  
23 pathway of bioreducible polymeric non-viral vectors <sup>40</sup>. Furthermore, non-viral nucleic acid  
24 carriers like for example cyclic Arginylglycylaspartic acid (RGD) peptide-conjugated polyplex  
25 micelles <sup>41</sup>, RGD-tagged and non-RGD-tagged PEGylated liposomes <sup>42</sup> and oligopeptide  
26 polyplexes <sup>43</sup>, were tracked and evaluated using labeled polynucleotides. Although most reports  
27 make use of fluorescently labeled pDNA, it should be noted that also the fluorescent labeling  
28 of mRNA is feasible with the label-IT reagents.

29 An alternative commercial nucleic acid labeling kit is available from Vector laboratories  
30 (Burlingame, Ca, U.S.A.) <sup>13</sup>. The FastTag Basic Nucleic Acid Labeling Kit is based on aryl-  
31 azide chemistry in which the universal disulfide containing FastTag reagent, when exposed to  
32 heat or light, becomes activated and attaches onto the nucleic acids without base specificity. In  
33 a second step, the disulfide is being reduced after which any sulfhydryl reactive moiety can be  
34 coupled. The FastTag system can react with any single or double-stranded DNA, RNA or  
35 oligonucleotide. For double-stranded nucleic acids, however, light-induced coupling is  
36 preferred over heat to avoid denaturation of the double-stranded nucleic acids structure. A wide  
37 variety of fluorescent tags and haptens can be added by using this method, mostly using  
38 maleimide-derivatives as sulfhydryl reactive moiety (see Table 1). The method is used for the  
39 same type of experiments as described for the Label-IT nucleic acid labeling kit, but their broad  
40 range of fluorophores, including far-red dyes, makes it possible to extend to *in vivo* applications.  
41 A series of poly(glutamic acid)-based peptide coatings were for example targeted to the liver,  
42 spleen, spine, sternum, and femur. DNA accumulation in these tissues was confirmed by  
43 AlexaFluor 680 - pDNA <sup>44</sup>. This versatility was also employed to make biotinylated plasmids

1 for *in vivo* transfection with Lac-PEI. Intracellular location was afterwards visualized in  
2 cryosections by adding streptavidin conjugated dyes <sup>45</sup>.

3 An older labeling kit is the ULYSIS<sup>®</sup> Nucleic Acid Labeling Kit (Molecular Probes, OR.,  
4 U.S.A.). AlexaFluor<sup>®</sup> fluorophores are linked to the bases of DNA, RNA, oligos and peptide  
5 nucleic acids (PNA) through the Universal Linkage System platinum-based chemistry. This  
6 system consists of a square planar platinum complex, stabilized by a chelating diamine. One of  
7 the two remaining coordination sites of this complex is occupied by a fluorophore, the second  
8 one will bind to the N<sup>7</sup> position of (deoxy)guanine <sup>14</sup>. This technique has been used to observe  
9 that the rate limiting step in pDNA-PEI transfections is the transfer from the lysosomal  
10 compartment to the nucleus <sup>46</sup>. Other carriers like mannosylated dendrimer/ $\alpha$ -cyclodextrin  
11 conjugates were evaluated <sup>47</sup> and to study how, and via which pathway, the DNA of a pathogen  
12 (*Cryptococcus neoformans*) stimulates dendritic cells AlexaFluor<sup>®</sup>647 was attached using the  
13 ULYSIS<sup>®</sup> kit <sup>48</sup>.

14 In summary, the covalent attachment of haptens and fluorophores offers a lot of benefits when  
15 random labeling of exogenous nucleic acids is desired. The different commercially available  
16 kits are readily available, easy to use and have already been extensively used for imaging  
17 polynucleotides and especially pDNA in living cells. A possible interaction with the system  
18 under study, however, should always be kept in mind, as each added fluorophore adds  
19 hydrophobicity and steric hindrance to the nucleic acids, when compared to their non-labeled  
20 analogs. Also, the often cationic fluorophores lower the overall negative charge of the  
21 polynucleotides which might interfere with the intracellular migration behavior of nucleic acids,  
22 complexation with and dissociation from cationic carrier.

## 23 Incorporation of modified nucleotides

24 Apart from the labeling strategies mentioned above, another popular commercially available  
25 option is the *in vitro* incorporation of modified nucleotides into the polynucleotide backbone.  
26 The term “modified” stands for a nucleotide that differs from the naturally occurring  
27 nucleotides through the addition of a functional moiety. At first, especially the nucleotides with  
28 radioactive atoms (<sup>32</sup>P, <sup>33</sup>P, <sup>14</sup>C, <sup>3</sup>H, <sup>35</sup>S) were used, but nowadays radioactive labels are often  
29 replaced by different types of coupling moieties, fluorescent labels (see Table 1) or haptens (see  
30 Table 2). Since the addition or replacement of nucleotides is a phenomenon that is occurring  
31 constantly in living cells, all these methods are based on naturally occurring enzymatic  
32 reactions.

33 In general, DNA or RNA synthesis is catalyzed by respectively DNA or RNA polymerases that  
34 read a DNA template in the 3' to 5' direction. The newly synthesized DNA or RNA molecules  
35 are then elongated in the 5' to 3' direction by the coupling of complementary nucleotides at the  
36 3' end. While nucleotide triphosphates (NTPs) are used in RNA molecules, DNA exists of  
37 deoxynucleotide triphosphates (dNTPs). In living cells, RNA polymerases are responsible for  
38 the transcription of mRNA from the genomic DNA. For labeling purposes, RNA polymerases  
39 (T7, SP6 and T3) manage the incorporation of modified NTPs into RNA by *in vitro* transcribing  
40 so-called complementary RNA from a provided DNA template. The addition of modified

1 nucleotides in the reaction mixture will therefore result in labeled RNA (see Figure 1 C) <sup>49-51</sup>.  
2 DNA polymerases are adaptations from the cellular enzymes that take care of DNA replication  
3 in every living cell. Also here, modified dNTPs can be incorporated during synthesis of the new  
4 DNA strand complementary to the template strand (see Figure 1 C). In other words, both RNA  
5 and DNA polymerases act in a template-dependent manner and incorporate modified  
6 (deoxy)nucleotides at random over the complete length of the polynucleotide chains.

7 There is also the possibility to incorporate modified nucleotides in a template-independent  
8 manner by using 'nick translation'. During nick translation, which is based on the cellular DNA  
9 repair mechanism, DNA is "nicked" by a DNA endonuclease creating single strand gaps in the  
10 nucleotide sequence. A DNA ligase, like DNA polymerase I, will start to repair the  
11 polynucleotide starting from this gap by addition of dNTPs supplied in the reaction mixture.  
12 Commercially available kits will mostly make use of DNase I as endonuclease which nicks ad  
13 random, while other sequence specific endonucleases can be bought separately. These  
14 endonucleases are derived from existing restriction enzymes, but modified to cut only one  
15 strand. When modified nucleotides are used, DNA fragments become fluorescently labeled (see  
16 Figure 1 C) <sup>52, 53</sup>. By controlling the ratio of modified over non-modified nucleotides in the  
17 reaction mixture, the labeling density of the resulting polynucleotides can be changed. Both  
18 methods described above incorporate modified nucleotides all over the polynucleotide chains,  
19 thereby achieving internal labeling of pDNA and mRNA. It is therefore to be expected, that  
20 modified nucleotides will also be present in the coding region of the pDNA or mRNA  
21 molecules. For some applications, this has to be avoided in which case it is better to add  
22 modified nucleotides only to the 3' or 5' end of the polynucleotides.

23 The addition of nucleotides to the 3' terminus of an existing polynucleotide can occur in a  
24 template-independent way by the use of terminal deoxynucleotidyl transferase (TdT). TdT has  
25 the strongest affinity for single-stranded DNA or the 3' overhang of double-stranded DNA and  
26 is used frequently for adding modified dNTPs to PCR primers or restriction enzyme generated  
27 3' overhangs (see Figure 1 C) <sup>54, 55</sup>. An example is the 3' EndTag DNA end labeling kit from  
28 Vectorlabs, which couples SH-GTP to the 3' end of DNA molecules. The SH-GTP can  
29 subsequently be modified with thiol-reactive molecules such as for example maleimide-coupled  
30 dyes. T4 RNA ligase is another enzyme that is able to add modified NTPs to the 3' terminus in  
31 a template-independent manner but only for RNA (see Figure 1 C). It should be noted that a  
32 DNA or RNA primer which contains a modified nucleotide at the 3' end (generated using TdT  
33 for example), can be used by DNA or RNA polymerases to be further elongated. Hence,  
34 internally labeled polynucleotides can also be formed in this way. 5' EndTag nucleic acid  
35 labeling kits are another available option from Vectorlabs, using T4 polynucleotide kinase to  
36 transfer a thio-phosphate from ATP $\gamma$ S to the 5' end of DNA, RNA or oligonucleotides. Again,  
37 any thiol-reactive molecule can be coupled to the 5' end of these polynucleotides. DNA or RNA  
38 primers containing a modified nucleotide at the 5' end, can also be used by DNA or RNA  
39 polymerases to create longer 5' end labeled polynucleotides (see Figure 1 C). Another  
40 interesting enzyme for end-labeling is the DNA polymerase I Klenow Fragment which lacks 5'  
41 to 3' exonuclease activity, but retained its 3' to 5' exonuclease activity and 5' to 3' polymerase  
42 activity. The 5' to 3' polymerase activity can be used to fill-in 5' overhangs (created for example

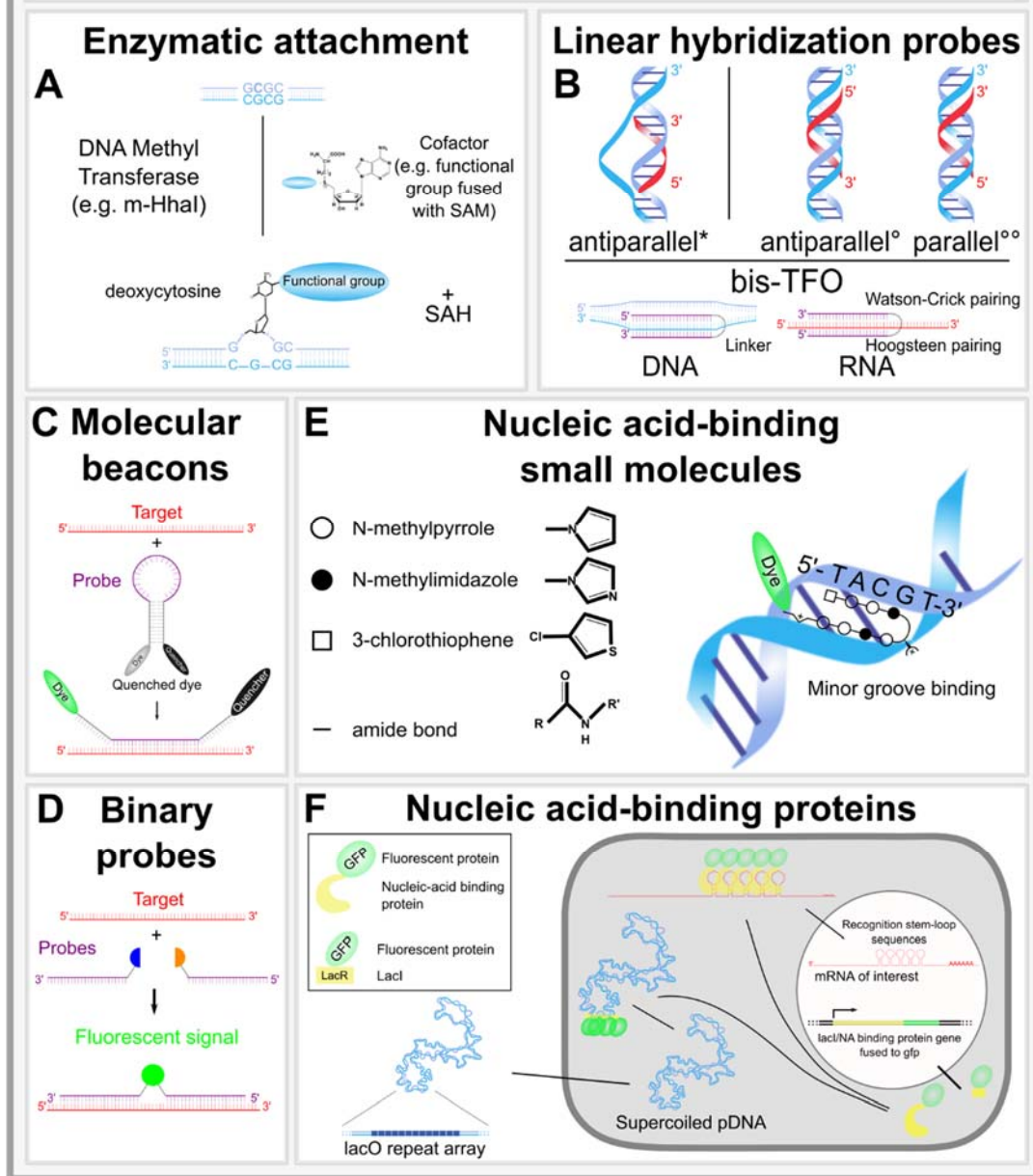
1 by restriction enzymes) with dNTPs, leading to labeled fragments when modified nucleotides  
2 are used.

3 As already indicated above, nucleotides can be modified in a broad range of ways (see Table  
4 1). In general, however, they can be classified as i) containing a fluorophore, ii) containing a  
5 hapten such as biotin or digoxigenin, iii) being alkyne- or azide-modified for click-chemistry  
6 based labeling or iv) being amine-modified for coupling to NHS-activated molecules (see Table  
7 1). Available fluorescent nucleotides are mostly UTP and CTP and their deoxy-analogues dUTP  
8 and dCTP. AlexaFluor<sup>®</sup> 488 and AlexaFluor<sup>®</sup> 594-dUTP were used during amplification of  
9 linear DNA fragments which were microinjected in the cytoplasm and nucleus of living cells.  
10 Their location and migration was followed using live-cell fluorescence microscopy <sup>56</sup>.  
11 Biotinylated nucleotides have been proven useful to conjugate streptavidin-quantum dots to  
12 pDNA for live-cell imaging. Biotinylated nucleotides were added in three different ways: nick  
13 translation, TdT addition and by end-filling by the DNA polymerase I Klenow Fragment <sup>57</sup>.  
14 The addition of “clickable” nucleotides has been documented as well. Copper-catalyzed click  
15 chemistry refers to coupling of an azide-containing molecule A with an alkyne-containing  
16 molecule B to form an A-B conjugate. Alkyne- and azide-modified nucleotides can be  
17 combined with a wide variety of respectively azide- and alkyne-modified dyes (see Table 1). It  
18 should be noted that copper ions damage DNA, resulting in for example strand breaks.  
19 Therefore, copper(I)-stabilizing ligands should be used, or alternatively, copper-free click  
20 reactions involving the coupling of dibenzocyclooctyne (DBCO)-containing molecules to  
21 azide-containing molecules could be considered. Attaching fluorophores to nucleotides, added  
22 by TdT, has been shown feasible via copper-catalyzed azide-alkyne cycloaddition, strain-  
23 promoted azide-alkyne cycloaddition, Staudinger ligation or Diels-Alder reaction with inverse  
24 electron demand <sup>58</sup>. Additionally, amine-modified (d)UTP, (d)CTP and (d)ATP are available,  
25 enabling the coupling of NHS-activated fluorophores to respectively DNA (dNTPs) and RNA  
26 (NTPs) (see Table 1).

27 In summary, the addition of modified nucleotides is a versatile way of adding radioactive labels,  
28 fluorescent labels or functional moieties to a polynucleotide. The availability of commercial  
29 kits also makes them accessible to a broad audience although a well-equipped biotechnology  
30 lab and accompanying lab experience is a prerequisite.

## 31 **Labeling methods for sequence-specific attachment of fluorophores** 32 **to polynucleotides**

# Sequence-specific methods



1  
2 **Figure 2 Overview of sequence-specific labeling methods. (A) General principle of MTase directed**  
3 **fluorescent labeling, (B) Schematic of linear hybridization probe interactions and bis-triplex**  
4 **forming oligonucleotides, \*: antiparallel interaction following Watson-Crick base pairing, °:**  
5 **antiparallel interaction following reverse Hoogsteen base pairing, °°: parallel interaction**  
6 **following Hoogsteen base pairing (C) Principle of molecular beacons, (D) Principle of binary**  
7 **probes (E) The principle of nucleic-acid binding small molecules based on a structure described**  
8 **by Nickols et al. (F) The mechanism of GFP-tagged nucleic acid-binding proteins and the**  
9 **lacO/LacI principle. SAM = S-Adenosyl-L-methionine, SAH = S-Adenosyl-L-homocysteine, NA:**  
10 **Nucleic Acid, TFO: Triplex forming oligonucleotide, R and R': functional moieties**

1 Sequence-specific labeling techniques comprise every method that labels polynucleotides at a  
2 predefined primary sequence. The primary sequence of polynucleotides is determined by the  
3 particular way in which each of only 4 possible nucleotides is ordered. Targeting specific  
4 sequences is beneficial to obtain site-specific labeling, without (much) off-target binding. From  
5 a labeling point-of-view this opens up a plethora of opportunities with regards to selectivity,  
6 the choice of labeling density and label location. The first technique that is described is  
7 remarkably similar to the random covalent attachment methods. The sequence-specificity lies  
8 in the fact that an enzymatic reaction is used, instead of the chemical reactions as shown above,  
9 adding functional groups only to those sequences that are recognized by the respective enzymes.

## 10 Enzymatic labeling at a specific target sequence

11 In enzymatic labeling, a functional group (or fluorophore) is added to nucleic acids due to an  
12 enzyme-mediated reaction between the nucleic acid and a dedicated co-factor. This direct  
13 modification of the chemistry of the nucleobase by the enzyme, distinguishes this method from  
14 those that use enzymes to incorporate modified nucleotides as mentioned above.

15 The enzymes used in the so-called Sequence-specific Methyltransferase-Induced Labeling  
16 (SMILing) method are DNA methyltransferases (MTases) <sup>60</sup>. The adenine-specific DNA  
17 MTase from *Haemophilus haemolyticus*: M.HhaI for example recognizes the double-strand  
18 DNA sequence 5'-GCGC-3' and subsequently catalyzes a covalent bond formation between  
19 the activated methyl group from a specifically designed co-factor (e.g. S-adenosyl-L-  
20 methionine) to the exocyclic amino group of cytosine (see Figure 2 A). The choice of MTase  
21 will determine the recognition sequence at which functional groups are attached. At the  
22 moment, most recognition sequences exist of 4 to 6 base pairs in which nucleophilic attack will  
23 occur on Adenine, Guanine or Cytosine. The co-factor is however equally important, as it  
24 determines the functional group which is added in the enzymatic reaction. Synthesis of new co-  
25 factors determines the range of molecules that can be attached to the activated group <sup>61</sup>.  
26 Originally this method was used to add methyl groups, but linear alkyl, alkenyl, and alkynyl  
27 functionalization was developed opening up more possibilities <sup>60, 62, 63</sup>. In the meanwhile a  
28 similar approach was developed for RNA <sup>64, 65</sup>. Based on these techniques the mTAG method  
29 was developed, making it possible to incorporate a primary amine group, which can be used for  
30 chemoligation reactions with all amine-reactive probes (e.g. NHS coupled-dyes, see Table 1 for  
31 examples) onto DNA or RNA <sup>66</sup>. For an in depth overview of the progress in this field the  
32 review by Gillingham and Shahid is recommended.

33 This enzymatic labeling approach is interesting because it enables labeling polynucleotides  
34 internally in a sequence-specific manner. Especially for RNA, which is mostly fluorescently  
35 labeled using 3' or 5' end labeling, this might be an interesting opportunity. Labeling density  
36 can be easily controlled by designing polynucleotides that contain a fixed number of recognition  
37 sequences. Labeling position, on its turn can be tuned by incorporating recognition sequences  
38 side-by-side, for example flanking the encoding region of the polynucleotide of interest. Also,  
39 by combining different enzymes with their own recognition sequence and specific co-factor,  
40 dual-labeling of polynucleotides on pre-defined sites is possible. Despite these interesting  
41 features, only a limited amount of studies have employed enzymatic labeling for live-cell

1 imaging applications. Schmidt et al. evaluated the transfection of enzymatically labeled pDNA  
2 with a non-viral carrier. Imaging was however performed in fixed cells. Due to the absence of  
3 a commercial supplier, follow-up publications in living cells are still lacking. Nevertheless,  
4 research is still ongoing to further optimize this labeling method<sup>69</sup>. Neely et al. for example  
5 adapted this technique to create a DNA fluorocode, making use of the sequence specificity.  
6 Fluorophores are added at different locations depending on the location of the recognition  
7 sequence which will differ depending on the target nucleic acid. The specific pattern of  
8 fluorophores on the target can then be used as a barcode for fast nanometer scale DNA sequence  
9 information<sup>70, 71</sup>. Along this line of thought enzymatic labeling is also used for rapid  
10 bacteriophage strain typing<sup>72</sup>.

11 In summary, enzymatic labeling is a promising covalent labeling technique which adds  
12 sequence specificity when compared to the random methods. The translation to a broad  
13 audience will however benefit from the development of a straight-forward labeling kit, ruling  
14 out the need for a thorough understanding of the enzymatic reaction before it can be employed.

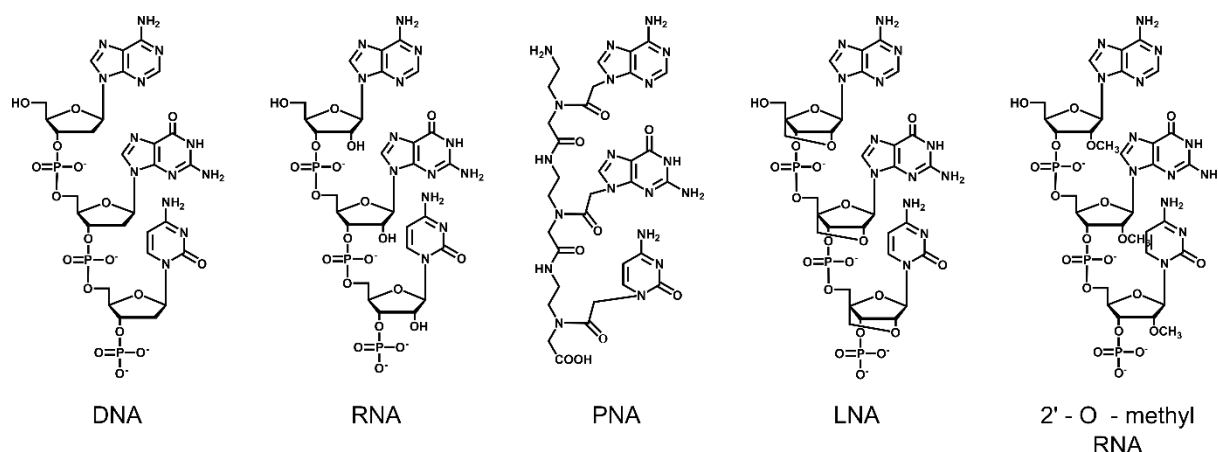
## 15 Nucleic acid-based hybridization probes

16 The next method that will be discussed makes use of oligonucleotides which recognize and bind  
17 to their nucleic acid target based on sequence complementarity. Therefore, knowing the exact  
18 sequence of the target polynucleotide is key. Sequence complementarity can manifest itself in  
19 two types of interactions: antiparallel and parallel to a nucleic acid strand. Antiparallel  
20 interactions follow Watson-Crick or reverse Hoogsteen base pairing, while parallel interaction  
21 makes use of Hoogsteen base pairing. For single strand DNA and RNA, the complementary  
22 oligonucleotides will prefer to follow an antiparallel interaction following Watson-Crick base  
23 pairing. For double stranded DNA, antiparallel interactions following Watson-Crick base  
24 pairing is also the most common interaction, but it requires the displacement of one strand to  
25 form a new oligo-DNA duplex (see Figure 2 B). Oligonucleotides that interact in an antiparallel  
26 manner following reverse Hoogsteen base pairing, as well as oligonucleotides interacting in a  
27 parallel manner, mainly bind into the major groove of the double helix, creating a triple helix  
28 structure at the binding site<sup>73</sup> (see Figure 2 B). These oligonucleotides regardless of their  
29 orientation are called triplex forming oligonucleotides.

## 30 **Linear oligonucleotide probes**

31 Already in the late seventies, it was proposed that antisense oligonucleotides could have a  
32 potential in a therapeutic environment<sup>74</sup>. In this method, short DNA or RNA oligonucleotides  
33 are designed to bind antiparallel to complementary mRNA sequences inducing downregulation  
34 of this specific mRNA. When a fluorescent label is attached to these oligos, this binding could  
35 be visualized. A well-known example where the principle of nucleic acid-binding  
36 oligonucleotide probes is used, is in the cytogenetic technique fluorescence in-situ hybridization  
37 (FISH) where the location of a target DNA or RNA sequence is visualized in fixed and  
38 permeabilized cells<sup>75</sup>. This is achieved by the addition of fluorescently labeled oligonucleotide  
39 probes, which bind to their complementary sequence and can be visualized by fluorescence  
40 microscopy<sup>76, 77</sup>.

1 This approach was also applied in live-cells, but low stability and poor cell uptake of these  
 2 DNA and RNA oligonucleotide probes led to the development of modified oligonucleotides<sup>78</sup>.  
 3 A combination of backbone and sugar moiety modifications proved to be the most interesting,  
 4 since binding affinity is less affected compared to modifying the bases themselves<sup>79</sup>. Peptide  
 5 (or polyamide) Nucleic Acid (PNA)<sup>80, 81</sup>, Locked Nucleic Acid (LNA)<sup>82</sup> and 2'-O-methyl and  
 6 2'-O-aminoethyl chemistry based nucleic acids<sup>83, 84</sup> are some examples of modified  
 7 oligonucleotides used to create a strong and sequence-specific binding to complementary  
 8 sequences (see Figure 3).



9

### 10 **Figure 3 Structures of DNA, RNA and the backbone-modified oligonucleotides**

11 Probably the best known modified oligonucleotides are PNAs, where the backbone consists of  
 12 N-(2-aminoethyl)-glycine units linked by amide bonds. The purine (A, G) and pyrimidine (C,  
 13 T) bases are attached to the backbone through methylene carbonyl linkages (see Figure 3)<sup>85</sup>.  
 14 This neutral backbone structure leads to a PNA/DNA or PNA/RNA interaction which is  
 15 stronger than just the DNA/DNA or RNA/RNA interaction. Furthermore, this interaction is also  
 16 more sequence-specific. Interactions can be either, the preferred, antiparallel method, which  
 17 requires strand displacement and follows Watson-Crick base pairing or parallel pairing which  
 18 creates a triple helix. The combination of both can be found in bis-PNA (2 PNA) which is  
 19 therefore a triple helix forming oligonucleotide (PNA-DNA-PNA) (see Figure 2 B). A bis-PNA  
 20 – DNA triplex was the first PNA complex described and it is probably the most interesting with  
 21 regards to labeling<sup>80</sup>. It consists of a Watson-Crick PNA strand coupled with flexible ethylene  
 22 glycol type linkers to the Hoogsteen PNA strand (see Figure 2 B). Often the Hoogsteen strand  
 23 is optimized by replacing cytosine with pseudoisocytosine, eliminating the need for a low pH  
 24 during hybridization<sup>86</sup>. The versatile use of PNAs has been excellently reviewed by Nielsen  
 25 and Egholm.

26 LNA bases are far more identical to DNA (or RNA) bases compared to the modifications seen  
 27 in PNA bases. The only change from a DNA (or RNA) base is the introduction of an additional  
 28 2'-C, 4'-C-oxymethylene linkage that effects conformational fixation of the furanose ring in a  
 29 C3'-endo conformation (see Figure 3)<sup>87, 88</sup>. Oligonucleotides consisting of only LNA bases  
 30 however have no double helix binding capacity<sup>89, 90</sup>. LNA-containing oligonucleotides on the  
 31 other hand undergo Watson-Crick base pairing resulting in thermostable, 3'-exonucleolytic  
 32 stable and sequence-specific double helix interactions with DNA and RNA<sup>82, 87, 91</sup>. Bis-LNA



1 was also developed more recently to bind to supercoiled DNA in a fashion similar to bis-PNA  
2 (see Figure 2 B) <sup>92</sup>.

3 2' O-aminoethyl modified RNAs differ only very slightly from regular RNA in that they have  
4 a short amino alkyl group attached to the 2' position of the ribose which allows a specific  
5 charge-charge contact of this protonated amino group (see Figure 3) with a proximal phosphate  
6 group of the DNA duplex. This interaction strongly enhances the binding affinity of this  
7 oligonucleotide towards double-stranded DNA <sup>83</sup>.

8 Although originally designed to interfere with the nucleic acid metabolism, similar to antisense  
9 oligonucleotides <sup>93</sup>, modified oligonucleotides can be used as a sequence-specific labeling  
10 strategy due to their specificity and strong nucleic acid-binding capacity. In general,  
11 fluorophores are attached via a linker to the 3' or/and 5' terminus of the oligonucleotide <sup>94</sup>,  
12 although fluorophore attachment is reported at all locations of the PNA strand through the use  
13 of fluorescently modified nucleotides during synthesis <sup>95</sup>. Other modified oligos can likely be  
14 internally labeled in a similar fashion. Early on the potential of PNAs in particular, was seen  
15 for detecting single base pair mutations by PNA-directed PCR, facilitating the amplification of  
16 mutants due to binding to the native templates and subsequently blocking of PCR amplification  
17 <sup>96</sup>. PNA strands employing a quencher and fluorescent reporter are also used to perform  
18 quantitative PCR (commercially available under the name of PANA qPCR, Panagene, Daejeon,  
19 Korea). Their high specificity makes them ideal candidates for multiplexing. As already  
20 mentioned, also for FISH modified oligonucleotides are the ideal candidates <sup>97, 98</sup>.

21 When we look at live-cell imaging, modified oligonucleotides have been used in very  
22 interesting applications. Molenaar et al. for example were the first to visualize the spatial  
23 localization and dynamics of telomeres in living human osteosarcoma cells, using fluorescently  
24 labeled PNA delivered to the cells using glass bead loading<sup>100</sup>. The same research group  
25 microinjected 2' O-methyl oligoribonucleotide-RNA probes and used photobleaching  
26 techniques to investigate the mobility of poly(A)<sup>+</sup> RNA throughout the nucleus <sup>101</sup>. These  
27 applications show the potential of modified oligos for intracellular labeling of endogenous  
28 nucleic acids in living cells. However, a major drawback is the background generated by the  
29 unbound oligonucleotides. Labeling of exogenously synthesized nucleic acid is also a  
30 possibility, which does not encounter this problem since a purification step is included before  
31 exposure to the cell. Zelphati et al., for example, followed the nuclear uptake of Rhodamine  
32 PNA-labeled nucleic acids and correlated Rhodamine-positive nuclei with green fluorescent  
33 protein (GFP) expressing cells. The PNA-based technique for labeling pDNA was also  
34 compared with random covalently labeled pDNA with regards to their postmitotic nuclear  
35 retention. It was seen that random covalently labeled pDNA was not retained within the nucleus  
36 of the daughter cells after cell division, whereas PNA-labeled pDNA acted as if it were  
37 unlabeled pDNA <sup>37</sup>. It was also established via atomic force microscopy and transfection  
38 experiments that pDNA labeled with quantum dots via PNAs was still functional <sup>103</sup>. Dual color  
39 labeling of one plasmid was later used in combination with confocal time-lapse imaging and  
40 intracellular trafficking by the same research group to study the intracellular DNA distribution  
41 and degradation after lipofection <sup>104</sup>. For the evaluation of non-viral carriers, PNA labeling is

1 also a good candidate as was shown by testing the effect cell-surface glycosaminoglycans have  
2 on the transfection efficiency of mixtures of low molecular weight PEI and cationic lipids <sup>105</sup>.

3 It should be noted that the GeneGrip PNAs and plasmids with corresponding PNA labeling sites  
4 (Gene Therapy Systems, U.S.A., now Genlantis, U.S.A.) are no longer commercially available.  
5 Obviously it is still possible to design and order vectors and PNAs with the appropriate  
6 sequences for site-specific labeling of pDNA. As with any oligonucleotide, however, a careful  
7 control of the annealing efficiency and the purification of non-bound oligos is necessary to  
8 obtain high quality and purified fluorescent nucleic acids.

9 Related to the direct labeling, as described until now, the concept of complementary sequence  
10 recognition by oligonucleotides is also recently used in live-cell RNA expression profiling. So-  
11 called Nanoflares (AuraSense, Skokie, IL, U.S.A.) are spherical nanoparticles with a gold core  
12 to which oligos complementary to the target RNA are attached. Via a second small  
13 oligonucleotide, a dye is bound on the complementary oligo closely to the gold nanoparticle  
14 whereafter it is quenched. When the Nanoflare enters the cell after receptor-mediated  
15 endocytosis, cytoplasmic target RNA can bind to the complementary oligo, effectively  
16 releasing the small oligo with the dye. This release induces unquenching whereafter this signal  
17 can be visualized via live-cell fluorescence microscopy, making it possible to quantify RNA  
18 synthesis <sup>106, 107</sup>. Building further on this concept, Briley et al. designed the so-called sticky  
19 flares. Instead of releasing the dye in the cytoplasm, the dye is attached to the target RNA via  
20 a long complementary oligo. This oligonucleotide is originally bound to one of the strands  
21 attached to the gold nanoparticle. When encountering the target RNA in the cytoplasm, the  
22 labeled oligonucleotide will have a higher affinity for the target RNA than for the one bound to  
23 the nanoparticle, thus effectively labeling the RNA in situ.

## 24 **Molecular beacons**

25 ‘Molecular beacon’ (MB) is a collective term for all probes that recognize and bind to a  
26 particular cellular target, enabling visualization. In this review, the molecular beacons are an  
27 extension on the concept of oligonucleotide probes which are mentioned above. While the  
28 “traditional” oligonucleotide probes make use of 1 fluorescent label, MBs employ two (or more)  
29 labels, which are attached at the opposite ends and make use of the fluorescence resonance  
30 energy transport (FRET) principle, a quencher/dye principle or a combination of both (see Table  
31 2 for examples) to ensure that the labels remain in a dark state until they bind to the target.  
32 Hereby guaranteeing a low background signal from unbound probes, which is necessary for  
33 live-cell microscopy. The oligonucleotide is designed in a stem-loop structure which brings  
34 both functionalities in close proximity when no target sequence is encountered. Upon binding,  
35 the probe’s stem-loop structure is stretched and respectively the donor dye (in FRET MBs) or  
36 quenched dye will become visible (see Figure 2 C) <sup>109, 110</sup>. The concrete implementation of this  
37 general principle is variable due to the use of different fluorophores, quencher/dye pairs,  
38 oligonucleotide conformations and number of oligonucleotides used (multiplexing), etc. but all  
39 are based on the same concept.

40 According to Santangelo, a good MB for tracking intracellular RNA should have 4  
41 characteristics, namely: i) the importance of delivery to the right cellular compartment, ii)

1 affinity for the target RNA without disturbing the functionality, iii) sequence sensitivity and  
2 last but not least, iv) a beacon should be visible throughout biogenesis, transport, translation,  
3 and degradation pathways. These characteristics also pinpoint the 4 bottlenecks of this labeling  
4 method. For labeling intracellular targets, MBs need to be delivered to the cell since they are  
5 not taken up spontaneously. The techniques used to deliver MBs, like microinjection, passive  
6 uptake, cationic transfection or reversible cell membrane permeabilization, suffer from low  
7 efficiency and a high toxicity to the cells. Also, the stability of the MB and the used dyes over  
8 time is important, since most cellular applications need to image the target over a longer period  
9 of time without the loss of signal. Despite these challenges, MBs easily found their way in live-  
10 cell imaging, but are also used in quantitative PCR. The exonuclease activity of DNA  
11 polymerases will cleave the MBs and disconnect the donor/quencher from the acceptor dye/dye,  
12 whereupon a fluorescent signal can be detected and correlated to an increase in amplified DNA  
13 <sup>109, 112</sup>. In live-cell imaging, MBs are popular for tracking intracellular RNA <sup>113-115</sup>, tracking  
14 DNA <sup>116</sup> and to study protein-DNA interactions <sup>117, 118</sup>.

15 Similar to MBs, a different type of RNA oligos undergo a conformational change, when they  
16 bind to their target sequence. These RNA oligonucleotides, targeting specific RNA sequences  
17 (called RNA aptamers), can be integrated and expressed in a stable or transient manner in cells.  
18 The induced conformational change upon binding makes them accessible to small fluorescent  
19 protein-like molecules, which allow in situ labeling of the target RNA <sup>119</sup>. The design and  
20 synthesis of the small dye is crucial for brightness and specificity since it should only be  
21 activated by one specific RNA aptamer. Paige et al. coined the term Spinach for their RNA  
22 aptamer that in combination with their fluorophore resembled the spectral properties of GFP  
23 the most, allowing the localization and tracking of RNA in living cells. Improvements in  
24 brightness and specificity on this system have been developed (e.g. Spinach2 <sup>121</sup>, RNA mango  
25 <sup>122</sup>) and more are under development.

## 26 **Binary probes**

27 One of the alternative approaches that combines the structural properties of linear oligos and a  
28 light-up principle of MBs, is the use of binary probes. In general, this approach makes use of  
29 two oligonucleotides which are complementary to adjacent sequences of the target nucleic acid  
30 and each have a different molecule that needs to be joined together to emit fluorescence on their  
31 ends (see Figure 2 D). Standard, this is a fluorescence acceptor and a fluorescence donor which  
32 are each situated at the ends of a oligonucleotide. Only in the presence of the target, the acceptor  
33 dye will receive the energy to have a significant emission<sup>123</sup>. This method has been applied  
34 routinely in Real-time LightCycler PCR Technology<sup>124, 125</sup>.

35 Although this method in theory offers improvements on the high signal background signal of  
36 linear oligo probes by employing a light-up principle and offer less chance of non-specific  
37 binding by using two independent oligos, some drawbacks should be noted. First of all, the  
38 complexity of the design of the probes is even higher than for single linear oligos or MBs<sup>126</sup>.  
39 Secondly, the signal of the acceptor fluorophore might suffer from the noise of the cellular  
40 autofluorescence. Additionally, the donor and acceptor fluorophores should be chosen in order  
41 to avoid overlap between the spectra of emission of donor and acceptor, which would generate

1 false positives. Finally, the hybridization of two separate oligos is from a kinetic and a  
2 thermodynamic point of view less favorable<sup>123</sup>. Kolpashchikov as well as Guo et al. made  
3 overviews of the different approaches that were developed within the concept of binary probes  
4 to tackle these issues. The most interesting developments are the use of a Ruthenium complex  
5 as a fluorescence donor (see Table 1) together with an organic fluorescence acceptor<sup>127</sup>, the  
6 creation of a lanthanide chelate (see Table 1)<sup>128, 129</sup> and quenched autoligation<sup>130-132</sup> which was  
7 used for live-cell imaging of bacterial strains<sup>133</sup> or to visualize mRNA in living cells<sup>134</sup>.

8 In summary, oligonucleotide hybridization strategies, as listed above, are a great way of  
9 labeling extracellular administered polynucleotides, like those for gene replacement therapy,  
10 and intracellular endogenous polynucleotides. Their mechanism however requires a well  
11 thought-through design of probes as well as target, ensuring stable and specific labeling of the  
12 target sequence with no, to limited, disturbance of its function. To obtain sufficient signal to  
13 follow single nucleic acid molecules for example, not one but an array of recognition sequences  
14 is needed<sup>135</sup>. In combination with the initial cost and optimization needed, these methods might  
15 not be for every cell biologist who ventures into live-cell imaging, but will remain in specialized  
16 labs.

## 17 Nucleic acid-binding small molecules

18 In contrast to the oligonucleotides described above, which are making use of (modified) nucleic  
19 acid bases, synthetic small molecules are making use of polyamides to bind to DNA in a  
20 sequence-specific manner. This approach was pioneered by the Dervan group at CalTech who  
21 described in detail how the invention of these type of hairpin pyrrole-imidazole polyamides was  
22 conceived<sup>136</sup>. Starting from the structure of naturally occurring antibiotics netropsin and  
23 actinomycin, which were known to be A/T tract selective DNA minor-groove binders, synthetic  
24 hairpin polyamides were developed which showed minor-groove binding and a high sequence  
25 specificity (see Figure 2 E). Originally, this approach was aimed at creating synthetic molecules  
26 for therapeutic gene expression regulation. However, conjugations of these polyamides to  
27 fluorescent dyes were developed and shown to be effective in binding double stranded DNA<sup>137</sup>  
28 and in intracellular localization<sup>138</sup>, due to their uptake in living cells<sup>139</sup>. Vijayanthi et al.  
29 assembled practical information about dye-polyamide conjugates and summarized the progress  
30 in this field based on the fluorescent dye used. Most notably was the increase of biological  
31 information obtained due to the overlap of the biological activity of the polyamides in gene  
32 expression regulation and the monitoring of the fluorescent signal in real time. The sequence  
33 specificity made them ideal candidates for following DNA repeats in genomes of eukaryotic  
34 cells<sup>141, 142</sup>. Furthermore FRET was observed when combining two polyamide conjugates with  
35 either Cy3<sup>®</sup> or Cy5<sup>®</sup>, opening the door to the study of DNA-protein interactions by the gain or  
36 loss of the FRET signal<sup>143</sup>.

37 In summary, conjugates of hairpin pyrrole-imidazole polyamides and fluorophores have found  
38 applications as sequence-specific nuclear stains. Due to their gene regulatory properties in  
39 which interest was shown as a therapeutic<sup>144</sup>, these minor-groove binders can aid in the study  
40 of the mechanism of these therapeutics. The chemical synthesis, design and cost might be a  
41 hurdle to the application by a broader cell biology community and remains for specialized labs.

## 1 Nucleic acid-binding proteins

2 Most labeling techniques discussed so far, with the exception of the molecular beacons, the  
3 binary probes and polyamide conjugates, are employed to label exogenous DNA and RNA prior  
4 to delivering them to the target cells. In situ labeling of polynucleotides, is however an  
5 interesting option in those cases that only nucleic acids which are present in the cytoplasm or  
6 nucleus of the cells should be detected. The main principle of in situ labeling is that the target  
7 cells express (fluorescently tagged) proteins that bind specific to DNA or RNA sequences. An  
8 advantage of this method is that the polynucleotides are not modified during the early steps of  
9 the transfection pathway, ruling out potential label-induced artifacts until cytoplasmic or  
10 nuclear entry. This implies that information on the steps in the transfection pathway before  
11 endosomal escape is lacking when using in situ labeling of nucleic acids.

12 In the informative review by Tyagi, an overview is presented of the methods that are available  
13 to track intracellular RNA. The nucleic acid binding methods: MS2 system <sup>145</sup>, Bg1 system <sup>146</sup>,  
14 the  $\lambda$ N system <sup>147</sup>, poly(A)-binding proteins <sup>148</sup> and PUMILIO1 <sup>149</sup>, are all systems who share  
15 the same basic principle <sup>113, 150</sup>. A fluorescent protein (most often GFP, see Table 1) is fused to  
16 a RNA-binding protein which has a strong affinity for a certain RNA motif or sequence, which  
17 is mostly incorporated in the target RNA sequence as a stem-loop structure. This binding of the  
18 GFP-tagged protein, makes it possible to visualize RNA molecules intracellularly and can be  
19 used for endogenous mRNA tracking (see Figure 2 F) (e.g. <sup>151</sup>), as well as for exogenously  
20 delivered RNA. Especially in the field of RNA viruses, these techniques have proved to be  
21 valuable <sup>146, 152</sup>.

22 A similar approach is available for DNA. A well-known example is the Lac operon/Lac  
23 repressor (lacO/LacI) system in which the LacI-GFP protein binds to the lacO sequence (see  
24 Figure 2 F) <sup>153</sup>. Annibale and Gratton used the lacO/LacI-GFP approach to visualize DNA,  
25 while the MS2 system was used to visualize the transcriptional kinetics of the produced mRNA.  
26 Apart from using GFP-fusion proteins, two methods are commercially available which attach a  
27 protein tag to the target sequence of a nucleic acid. When a fluorescently labeled ligand is  
28 added, it binds the protein tag resulting in a fluorescently labeled protein. Both the SNAP tag  
29 method <sup>155</sup> by New England Biolabs (cited by 798 papers (Google Scholar, September 2015))  
30 and the HaloTag technology <sup>156</sup> by Promega (cited by 472 papers (Google Scholar, September  
31 2015)) make use of this principle. These methods have the advantage that a broad range of cell-  
32 permeable organic fluorophores can be utilized in comparison with the fusion of, traditionally  
33 less bright, fluorescent proteins to the DNA-binding protein of interest. Nevertheless, the option  
34 of directly fusing a fluorescent protein to the DNA-binding protein might be preferred in cases  
35 where for example unwanted binding events in the nucleus are observed when using the  
36 commercially available methods <sup>157</sup>. This was also a concern when Shimizu et al. microinjected  
37 DNA into the cytoplasm and nucleus of cells. That is why, next to the use of modified  
38 nucleotides to detect the short DNA fragments, they worked with lacO arrays to visualize their  
39 long DNA fragments.

40 Apart from the lacO/LacI system, other naturally occurring transcription factors like zinc-finger  
41 nucleases, CRISPR/Cas-based methods and transcription activator-like effectors (TALEs) are

1 used. This last type can be designed to recognize very specific DNA sequences, which makes  
2 them great tools for labeling <sup>158, 159</sup>. Their specificity made it for example possible to track major  
3 satellite DNA throughout the cell cycle <sup>160</sup>. The use of these rather complex techniques is up to  
4 this point not routinely utilized in live-cell imaging.

5 In summary, nucleic acid-binding proteins are an interesting option in the range of labeling  
6 techniques available to researchers (see Table 2), but are certainly not the most straight-forward.  
7 The availability of commercial options relieves this partly, but the need for a stable or transient  
8 expression of the fluorescent protein-tagged proteins poses an extra hurdle which lowers  
9 efficiency. Furthermore, the need for a well-designed target also complicates the experimental  
10 design.

Table 2 Advantages and disadvantages of the different labeling methods

Labeling method	Pros	Cons	Ideal for:	Available functional groups	Key ref.
<b>Intercalating dyes</b>	+ Easy to use kits available	- Off-target interactions	Counterstaining	Possibilities all over the visual spectrum (e.g. YOYO-1, TOTO-3)	<sup>18</sup>
	+ Fast + Large scale reactions + Light-up principle	- Negative effects on NA conformation - Low efficiency dyes	Quantification of cellular uptake		<sup>21</sup> <sup>9</sup>
<b>Covalent attachment</b>	+ Easy to use kits available	- Negative effects on transfection at high labeling density	Polynucleotide tracking	Label-IT: DIG, biotin, DNP, Cy <sup>®</sup> 3, fluorescein, Cy <sup>®</sup> 5, TM-Rhodamine, CX-Rhodamine FastTag: thiol-reactive reagents ULYSIS: Alexa Fluor <sup>®</sup> 488-546-594-647	<sup>12 36 13</sup> <sup>14</sup>
	+ Fast + Large scale reactions + Strong covalent attachment		Random labeling		
<b>Modified nucleotides</b>	+ Kits available	- Prior knowledge of labeling mechanism	End labeling	NHS ester + primary amine Maleimide + thiol Azide + Alkyne Biotin + streptavidin Intrinsically fluorescent labeled nucleotides (see Table 1)	<sup>50 52 55</sup>
	+ Broad range of possible methods + End labeling possible	- Labor intensive - Expensive modified nucleotides	Random labeling		
<b>Enzymatic labeling</b>	+ Sequence-specific (4 to 6 bp recognition sequence) + Easy to use + Strong covalent attachment + Fast	- No individual components for reaction commercially available - Extensive knowledge of chemistry of co-factors necessary - Small scale reaction - Expensive enzymes and co-factors	Polynucleotide tracking	All NHS ester-dyes and haptens (e.g. NHS ester-AlexaFluor <sup>®</sup> 488, NHS ester-AlexaFluor <sup>®</sup> 647, NHS ester-biotin )	<sup>66 68</sup>
<b>Linear oligos</b>	+ Highly sequence-specific + Strong attachment + Fast + Large-scale reactions + Low cost per reaction + Multiplexing possible	- No kit available (anymore) - Needs sequence information of target - Needs knowledge of oligo design - Optimization needed - High initial price of nucleotides	Polynucleotide tracking	Same options as for modified nucleotides (see Table 1)	<sup>79 81 85</sup>
<b>MBs</b>	+ Highly sequence-specific + Strong attachment + In situ labeling possible + Light-up principle + Versatile	- No kit available - Intracellular delivery needed - Sequence information on target needed - Custom oligo design - Optimization needed - High initial price of (modified) probe	Intracellular detection of specific polynucleotides	Virtually all commercial organic quencher-dye pairs available (e.g. TAMRA – 5'-FAM, Quasar 670 - Cy <sup>®</sup> 5)	<sup>109 111</sup>

<b>Binary probes</b>	<ul style="list-style-type: none"> <li>+ Highly sequence-specific</li> <li>+ Strong attachment</li> <li>+ In situ labeling</li> <li>+ Light-up principle</li> <li>+ Versatile</li> <li>+ Reduction of background</li> </ul>	<ul style="list-style-type: none"> <li>- No kit available</li> <li>- Intracellular delivery needed</li> <li>- Sequence information on target needed</li> <li>- Custom design for two adjacent oligos</li> <li>- Optimization needed</li> <li>- High initial price of (modified) probe</li> </ul>	Intracellular detection of specific polynucleotides	Virtually all commercial quencher-dye pairs available (e.g. TAMRA – 5'-FAM, Quasar 670 - Cy <sup>®</sup> 5, Ru(II) – Cy <sup>®</sup> 5)	<i>123, 126</i>
<b>NA-binding small molecules</b>	<ul style="list-style-type: none"> <li>+ Sequence-specific</li> <li>+ In situ labeling possible</li> <li>+ Spontaneous uptake by cells</li> <li>+ Versatile</li> </ul>	<ul style="list-style-type: none"> <li>- No kit available</li> <li>- Sequence information on target needed</li> <li>- Not all sequences can be targeted</li> <li>- Extensive knowledge of polyamide conjugate chemistry necessary</li> <li>- Expensive</li> </ul>	Intracellular detection of specific polynucleotides	Same options as for modified nucleotides (see Table 1)	
<b>NA-binding proteins</b>	<ul style="list-style-type: none"> <li>+ Kits available</li> <li>+ Sequence-specific</li> <li>+ In situ labeling</li> <li>+ Cell compartment specific labeling possible</li> </ul>	<ul style="list-style-type: none"> <li>- Extensive knowledge of FP-tagged proteins needed</li> <li>- Adaption of target with recognition sequences to facilitate binding of proteins</li> <li>- Stable or transient expression of FP-tagged protein in cell needed</li> <li>- Labor intensive</li> <li>- Expensive</li> <li>- Optical properties of FP</li> </ul>	Intracellular detection of specific polynucleotides in specific cell compartment	All available fluorescent proteins. Mostly (E)GFP	<i>113 150</i>



## Lighting up the intracellular delivery path of pDNA and mRNA polynucleotides

The choice of an optimal labeling strategy to visualize nucleic acids in living cells will greatly depend on the specific research questions. The labeling of native DNA and RNA in the context of analyzing gene expression profiles, elucidating primary and secondary structure and following epigenetic modifications and visualizing interactions with other cell components has been nicely reviewed by Boutorine et al.. Here, we aim to overview the labeling choices which are available for fluorescent labeling of pDNA and mRNA, in the context of following their intracellular uptake, endosomal escape, mobility, stability and distribution in living cells.

Polynucleotides such as pDNA and mRNA are prominently negative due to the anionic charge at the phosphate group of each nucleotide in the polymer sequence. Polynucleotides can be directly delivered into the cytoplasm or nucleus of the cells by microinjection or electroporation. They are however most frequently complexed with positively charged carriers to enable cellular uptake of the (net positive) complexes that are formed. These complexes are taken up by endocytosis, after which they should escape the endosomal compartment to prevent lysosomal degradation. Finally the complexes should release their cargo into the cytoplasm (mRNA) or nucleus (pDNA) of the cells <sup>162, 163</sup>.

The different steps of the intracellular delivery path contribute to the overall observation that polynucleotides delivery is rather inefficient, especially when non-viral nucleic acid delivery is concerned. Therefore, each step of the intracellular trafficking has been the subject of intensive investigation over the past years. Hereby, fluorescence microscopy remains the most widely applied tool to gather information, going from more common wide field or confocal fluorescence microscopy setups, to more advanced instrumentation such as spinning disk microscopy, single particle tracking, fluorescence correlation spectroscopy and superresolution microscopy <sup>164-167</sup>.

The choice of labeling technique needs to be incorporated early in experimental design, at the same time microscopy method, cell line, transfection method and type of polynucleotide (mRNA or pDNA) are chosen. Several aspects have to be considered when choosing a labeling strategy for mRNA and pDNA, such as the need for single versus dual color labeling, random versus sequence-specific labeling, labeling of the polynucleotides during *in vitro* synthesis or intracellular (in situ) labeling. The ease and cost of the labeling procedure is also a factor to consider.

When complexing fluorescently labeled polynucleotides with non-labeled carriers for example, single-colored complexes are formed. These single-colored complexes can be used to follow for example intracellular uptake and endosomal escape, as well as the intracellular location of the (fluorescent) nucleic acids. Also, the colocalization of (e.g. red) complexes with GFP-tagged (green) endosomal vesicles could be performed <sup>40, 165</sup>. One should always keep in mind, however, that the fate of the non-labeled complexation partner cannot be followed in this experimental setup. Moreover, certain carriers, such as PEI have the tendency to quench fluorescence upon complexation. In that case, fluorescence will only return once the

polynucleotides have dissociated from their carrier. This principle was elegantly explored to develop an endosomal escape assay based on the (de)quenching of small oligonucleotide fragments <sup>168</sup>. By simultaneously labeling different types of endosomal vesicles, the specific type of endosomes from which complexes are preferentially released has been recently identified <sup>169</sup>. Apart from single color-labeling, both the carrier and nucleic acids can be labeled with spectrally separated fluorophores to obtain dual-colored complexes <sup>170</sup>. In this way, both parts of the delivery complex can be tracked before and after dissociation of the nucleic acids from the carriers. Dual-labeling of the polynucleotides themselves is also an interesting option to monitor their stability in the intracellular environment, as discussed below.

The choice between random and sequence-specific labeling of polynucleotides will largely depend on the biological application under investigation. Random labeling frequently results in more fluorophores per polynucleotide, generating brighter polynucleotides and complexes (until quenching occurs), suited for use with basic fluorescence microscopes. The two fast and easy options for random labeling, are intercalation and covalent attachment of probes. After endosomal escape, however, intercalating dyes hold the risk of re-distribution to endogenous nucleic acids in the cytoplasm or nucleus of the cells. Random covalent attachment of fluorophores to polynucleotides, on the other hand, has been proven to interfere with the endosomal escape and transcription/translation properties of the labeled polynucleotides themselves. Therefore, both labeling methods are best suited to follow the first two steps (cellular uptake and identification of endosomal vesicles) of the transfection pathway.

Whenever the random presence of dyes on the polynucleotide backbone holds a risk of disturbing the natural function of the polynucleotides, sequence-specific labeling can be preferred. Both for mRNA and pDNA, target sequences can be chosen outside the coding region. While linear oligos, MBs and binary probes can in theory make use of the natural primary sequence of the polynucleotides, plasmids for sequence-specific labeling are mostly designed to contain a known number of target repeats to assure sufficient and specific binding. Alternatively, recognition site arrays for enzymatic labeling can be incorporated at the location of choice. An elegant application of sequence-specific labeling of polynucleotides is the spatially separated double labeling <sup>104</sup>. This allows the coding region to be flanked with for example a green and a red fluorophore, which can be of interest to monitor the stability of polynucleotides. Indeed, as long as the polynucleotides remain intact, the green and red color will colocalize. As soon as backbone degradation occurs the colocalization is expected to disappear. Despite many possible experimental designs, the disadvantage of the sequence-specific labeling methods remains the need to custom order and design your polynucleotide of interest. This requires the preparation, isolation and quality control of cloned pDNA vectors, which might be a challenge for researchers with a limited biotechnology background. It should be noted that for mRNA stability testing, 5' and 3' end labeling of the synthesized mRNA can also be considered, overcoming the need to incorporate recognition sequences into the template DNA used to prepare mRNA.

The labeling of polynucleotides during synthesis is an option that is of interest when fluorescently labeled mRNA molecules are desired. Indeed, the *in vitro* synthesis of mRNA is a routine method to prepare mRNA from a DNA template. Non-modified NTPs are frequently

replaced by modified non-fluorescent NTPs in the reaction mixture to yield more stable and less immunogenic mRNA <sup>171</sup>. The addition of labeled NTPs to prepare fluorescent mRNA is thus a slight and easy adaptation of the normal protocols. Also, fluorescent cap analogues are available for 5' end labeling <sup>172</sup>. 3' end labeling, on the other hand, would require fluorophore addition to the mRNA poly-A tail.

A final consideration to be made is the added value of using an in situ labeling method. For the detection of endogenously synthesized polynucleotides, in situ labeling is obviously the only suitable method to assure intracellular detection. In situ labeling of exogenously delivered polynucleotides could also be an interesting option to visualize the polynucleotides only after reaching the cytoplasm or nucleus of the cells. This would allow detection of endosomal escape events of initially non-labeled polynucleotides, ensuring that the labeling strategy cannot interfere with the escape process. The three possible methods for in situ labeling are the use of molecular beacons, nucleic acid-binding small molecules or the nucleic acid binding proteins. Molecular beacons have the advantage that they employ a light-up principle, thereby limiting any fluorescent background signal. The use of organic dyes also increases their brightness and photostability, compared to fluorescent proteins. A substantial disadvantage of these molecular beacons is the need for a delivery method since they are essentially (modified) oligonucleotides, which will not spontaneously reach the cytoplasm of cells. Mechanical methods like microinjection, electroporation and bioballistics as well as chemical methods like toxin-mediated cell membrane permeabilization, liposomes and polyplexes are possible strategies <sup>173-175</sup>. The nucleic-acid binding small molecules have the advantage of MBs in their use of organic dyes while being spontaneously taken up by cells. The need for an in depth knowledge of the polyamide conjugate chemistry, cost and the limitations in sequences that can be specifically recognized, limits their application by a broader audience. For in situ labeling with nucleic acid-binding proteins, the fact that one molecule of coding DNA/mRNA leads to multiple functional proteins, is an advantage when compared to the large amount of MBs and polyamide conjugates that need to be present for a good reporter signal. To accomplish this however, the target cells need a transient or stable expression of nucleic-acid binding proteins, which might be experimentally challenging. Furthermore, extra toxicity or cell stress that might influence the intracellular path of the polynucleotides under investigation might be induced.

It should be noted that apart from coupling fluorophores and haptens to polynucleotides, also the label-free detection of polynucleotides is gaining attention. The biggest advantage is that the use of bulky fluorophores or haptens is avoided, by employing the molecule's own chemical fingerprint for visualization. Interestingly, label-free detection can occur over a long time, since photobleaching, which is an important bottleneck in live-cell imaging, is no issue anymore. Raman scattering is one of those promising techniques that might be used to visualize nucleic acids <sup>176</sup>. Alkyne-bearing nucleotides were for example used to visualize DNA and RNA synthesis *in vivo* by stimulated Raman spectroscopy <sup>177</sup>. This principle might be extended to a priori labeling, making this an interesting option for the future.

## Conclusion

In this review, we aimed to overview different labeling strategies for adding fluorophores or haptens to polynucleotides such as pDNA and mRNA. Also, some considerations on how to evaluate the compatibility of a certain labeling technique for tracking polynucleotides in the context of non-viral gene delivery were made. It is clear that more than one labeling strategy might be suited for a given application. Then, ease of operation, cost and compatibility of the possible fluorophores with the research instrumentation will be valuable extra concerns to determine the preferred labeling option. Obviously, one should always carefully evaluate if the labeling method and labeling density of choice is not interfering with the process under investigation, relative to a non-labeled control. It is expected that the continuous development of advanced microscopy methods will further shape the future of polynucleotide labeling strategies. One exciting new application, for example, is the label-free detection of molecules, which is being explored in a broad range of fields. Undeniably, live cell imaging will continue to play an important role in unraveling intracellular mysteries, creating a step-by-step insight in (and possible optimization of) the polynucleotide delivery pathways.

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## **Abbreviations**

- pDNA: plasmid DNA
- mRNA: messenger RNA
- NA: nucleic acid
- PCR: Polymerase Chain Reaction
- PEI: PolyEthyleneImine
- RGD: Arginylglycylaspartic acid
- PNA: Peptide Nucleic Acid
- NTP: Nucleotide Triphosphate
- dNTP: deoxyNucleotide Triphosphate
- TdT: Terminal deoxynucleotide Transferase
- MTase: MethylTransferase
- FISH: Fluorescence In Situ Hybridization
- LNA: Locked nucleic acid
- MB: Molecular Beacon
- FRET: Fluorescence Resonance Energy Transport
- FP: Fluorescent Protein
- GFP: Green Fluorescent Protein

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