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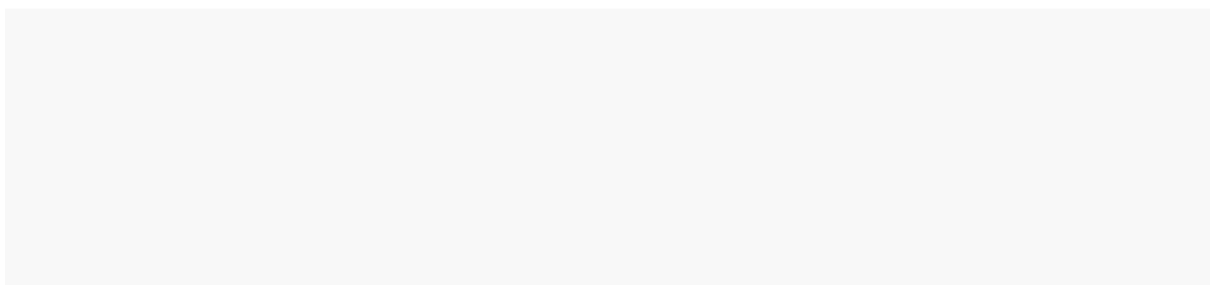

Fluorescent labeling of plasmid DNA and messenger RNA: gains and losses of current labeling strategies

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Abstract

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

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

Introduction

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

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

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

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

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

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

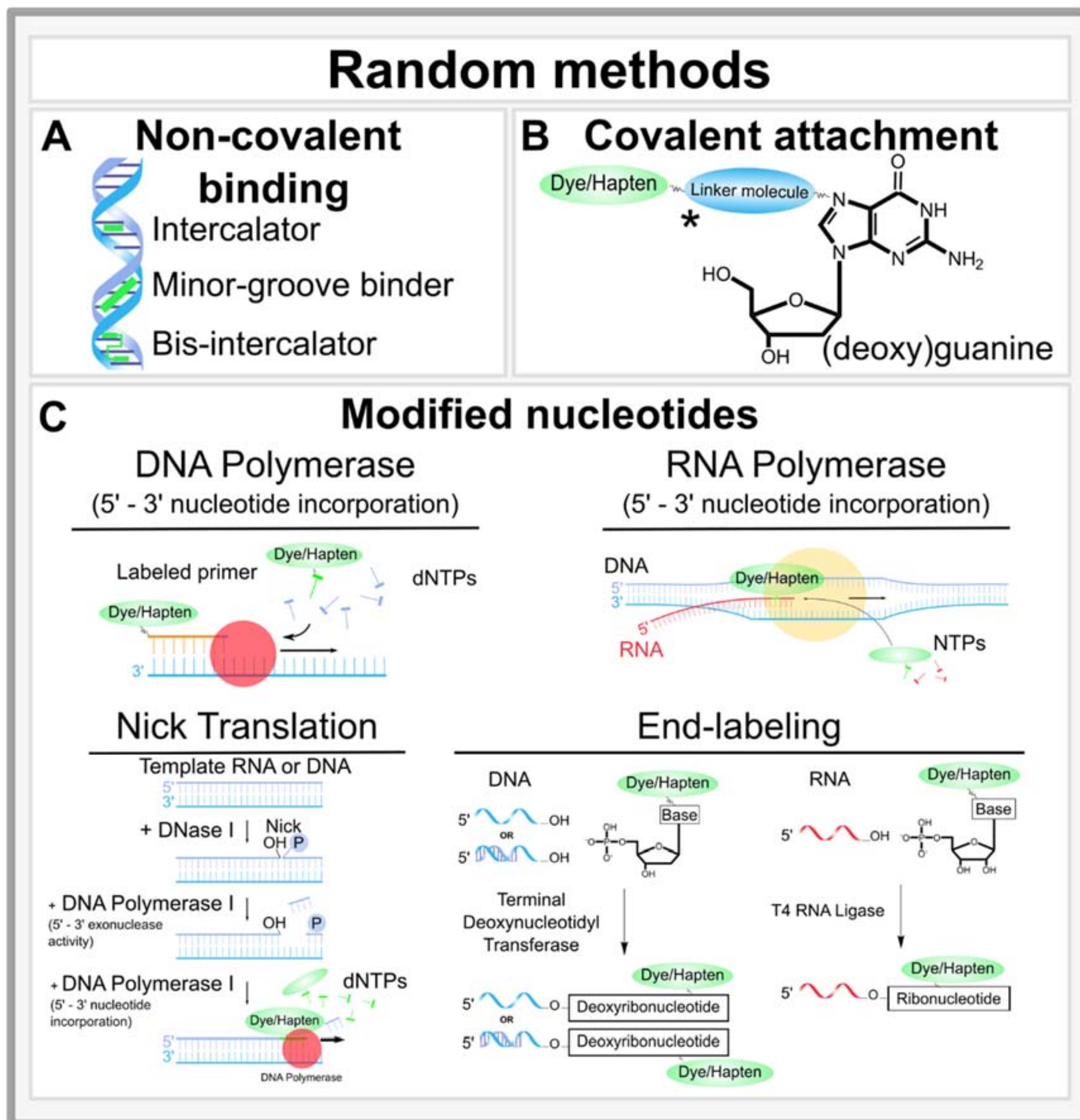
Fluorescent dye class		Functionalities	Photophysical properties				Supplier
			$\lambda_{Abs.}$ (nm)	$\lambda_{Em.}$ (nm)	Quantum yield	Remarks	
Cyanine (Cy®) dyes	Cy®3	SE, CA, strep, mal, azide, alkyne, NH_2, UTP, CTP, GTP, ATP, pCp, dUTP, dCTP	550	570	0.15	Broad range of applications, less bright than Alexa Fluor® 546	Invitrogen, Jena Bioscience, GE Healthcare Life Sciences, Lumiprobe, …
	Cy®5		649	670	0.27	Broad range of applications, less bright than Alexa Fluor® 647	
Cyanine dimers	YOYO-1	N/A	491	509	0.38†, 0.25‡[7]	Photocleavage of NA and bleaching reported[8]	Molecular Probes™
	TOTO-3		642	660	0.06	Bleaches fast (20-30s)[9]	
Ethidium bromide		N/A	285	605	0.20	Especially used for gel staining, but suspected mutagenicity	Invitrogen, Sigma-Aldrich, Bio-Rad, …
Alexa Fluor®	Alexa Fluor ®488	SE, CA, strep, mal, azide, alkyne, UTP, dUTP	495	519	0.92	Fluorescence output unmatched by any other spectrally similar dye	Molecular Probes®
	Alexa Fluor ®546		556	573	0.79	Cy®3 and TM-Rhodamine substitute	
	Alexa Fluor ®594	SE, CA, strep, mal, azide, alkyne, dUTP	590	617	0.66	Bright and photostable	
	Alexa Fluor ®647	SE, CA, strep, mal, azide, alkyne, ATP, dUTP, dCTP	650	668	0.33	brighter substitute for Cy®5	
ATTO dyes	ATTO 488	SE, CA, strep, mal, azide, alkyne, NH_2, biotin, UTP, ATP, dUTP, dCTP	501	523	0.80	Very high photostability	ATTO-TEC, Sigma-Aldrich, Jena Bioscience
	ATTO 647N		644	669	0.65	Cy®5 substitute, high thermal and photostability, exceptionally high stability towards atmospheric ozone	
Fluorescein	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™, Sigma-Aldrich, Jena Bioscience, …

	5-FAM (carboxylic acid)	SE, azide, alkyne, strep, NH_2, dUTP, dCTP	494	518	0.90	SE form more stable bond with amines compared to FITC-SE	Molecular Probes™, Sigma-Aldrich, Jena Bioscience, Lumiprobe, …
TM-Rhodamine	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_2, dUTP, dCTP	555	580	0.10	More stable than TRITC	Molecular Probes™, Sigma-Aldrich, AnaSpec.
CX-Rhodamine	5-ROX	SE, mal, azide, alkyne	576	591	0.70 (PO_4 buffer, pH 9)	Unstable compared to other rhodamines	Molecular Probes™, Sigma-Aldrich, Jena Bioscience, AnaSpec.
Fluorescent proteins	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®
Quantum dots	CdSe-ZnS	CA, strep, mal, azide, alkyne (cyclooctyne), NH_2, biotin			0.20-0.50*	Bright, photostable, broad excitation, tunable narrow emission, but concerns about cell toxicity	Molecular Probes™, Sigma-Aldrich, Mesolight
Lanthanide chelate	Eu(III)-chelates	SE, CA, strep, mal, azide, alkyne, NH_2, biotin	376	616	~1 (^2H_2O[10])	Narrow emission peaks, long fluorescence lifetimes, high photostability but often needs antenna to transfer absorbed excitation energy to the lanthanide	PerkinElmer,
	Tb(III)-chelates		340	545			Invitrogen, PerkinElmer
Transient metal complexes	Pt(II), Ir(III), Ru(II), Re(I)based	SE, CA, strep, mal, azide, alkyne, NH_2, 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_2: amine, †: double strand DNA, ‡: single strand DNA, *: dependent on shell thickness[11], FLIM: Fluorescence-Lifetime Imaging Microscopy

1

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[15], but it is believed
5 most of them exhibit only one of the two binding modes (e.g. YOYO-1[16]), but the combination
6 of both has also been reported for PicoGreen®[17].

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 α-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[18]. 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[19]. 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[20].

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, π-stack interactions (intercalated moiety), hydrogen-bonding,
26 van der Waals interactions, hydrophobic interactions and steric hindrance effects all play a
27 role[21]. 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 [9].

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 [22])(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

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

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

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

Random covalent attachment of fluorophores and haptens

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

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

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

for *in vivo* transfection with Lac-PEI. Intracellular location was afterwards visualized in cryosections by adding streptavidin conjugated dyes [45].

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

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

Incorporation of modified nucleotides

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

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

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

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

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

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

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

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

Labeling methods for sequence-specific attachment of fluorophores to polynucleotides

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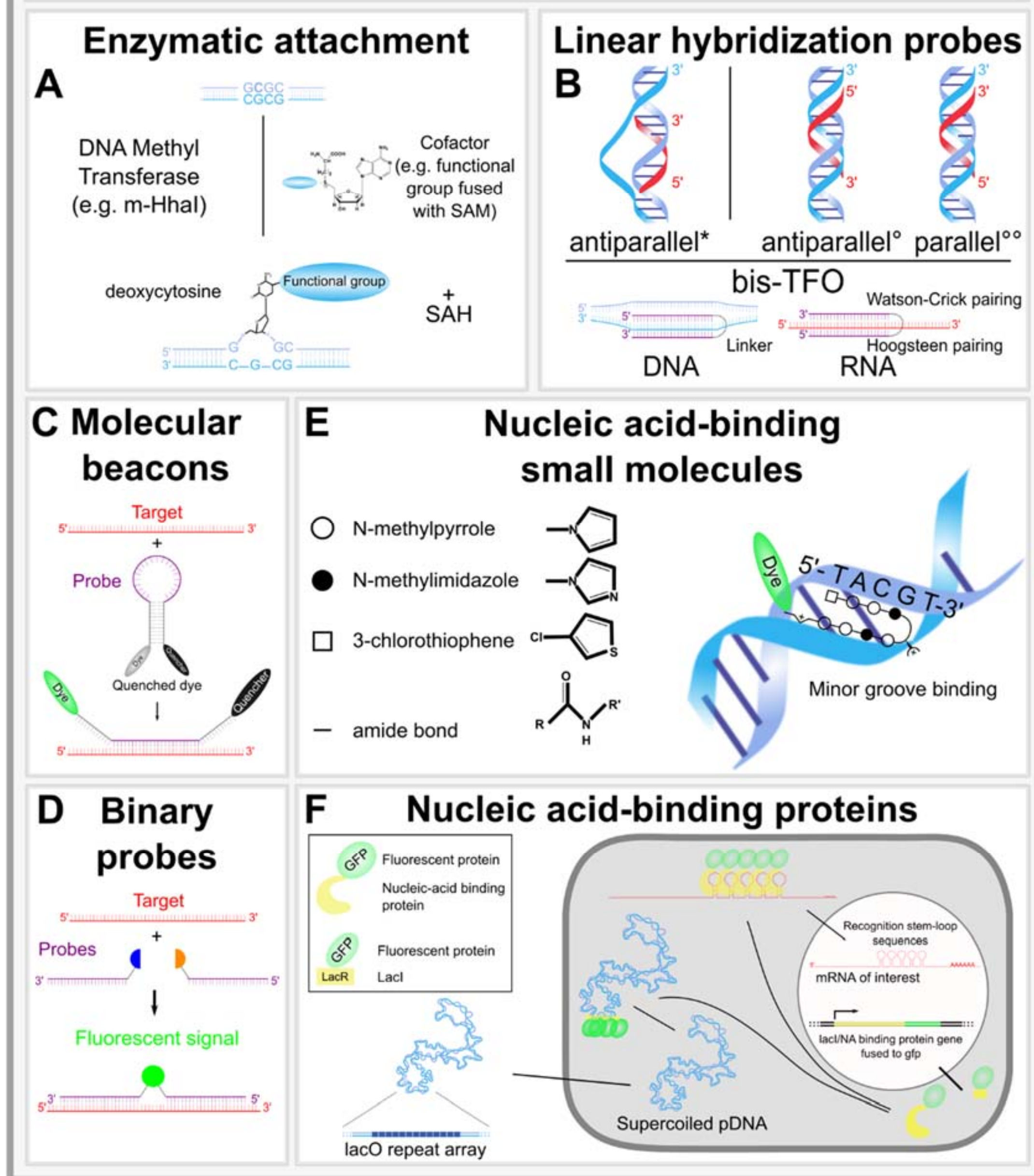

2 **Figure 2 Overview of sequence-specific labeling methods. (A) General principle of MTase directed**
3 **fluorescent labeling, (B) Schematic of linear hybridization probe interactions and bis-triplex**
4 **forming oligonucleotides, *: antiparallel interaction following Watson-Crick base pairing, °:**
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**

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

Enzymatic labeling at a specific target sequence

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

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

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

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

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

Nucleic acid-based hybridization probes

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

Linear oligonucleotide probes

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

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

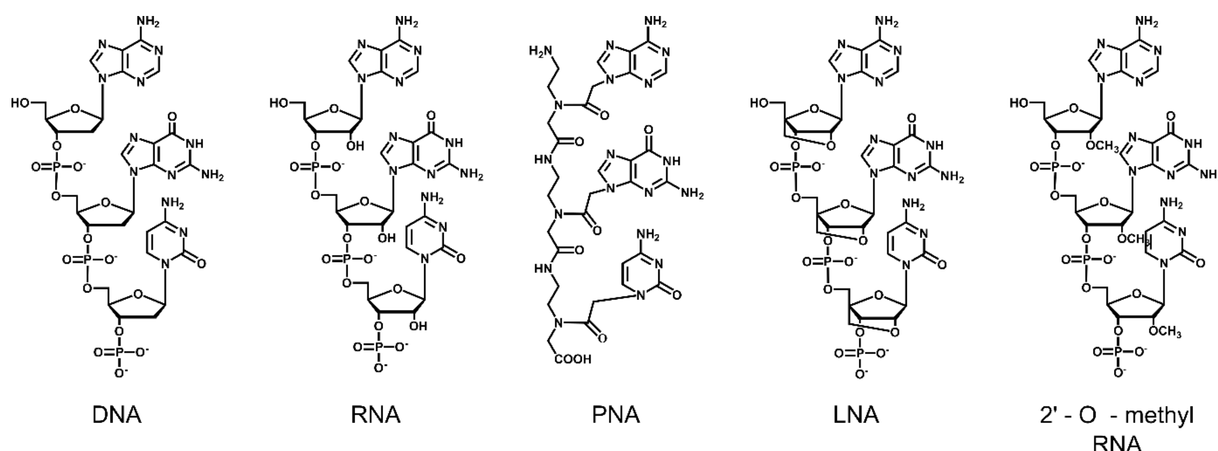

Figure 3 Structures of DNA, RNA and the backbone-modified oligonucleotides

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

LNA bases are far more identical to DNA (or RNA) bases compared to the modifications seen in PNA bases. The only change from a DNA (or RNA) base is the introduction of an additional 2’-C, 4’-C-oxymethylene linkage that effects conformational fixation of the furanose ring in a C3’-*endo* conformation (see Figure 3) [87, 88]. Oligonucleotides consisting of only LNA bases however have no double helix binding capacity [89, 90]. LNA-containing oligonucleotides on the other hand undergo Watson-Crick base pairing resulting in thermostable, 3’-exonucleolytic stable and sequence-specific double helix interactions with DNA and RNA [82, 87, 91]. 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) [92].

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 [83].

8 Although originally designed to interfere with the nucleic acid metabolism, similar to antisense
9 oligonucleotides [93], 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 [94],
12 although fluorophore attachment is reported at all locations of the PNA strand through the use
13 of fluorescently modified nucleotides during synthesis [95]. 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 [96]. 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 [97, 98].

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[100]. The same research group
25 microinjected 2' O-methyl oligoribonucleotide-RNA probes and used photobleaching
26 techniques to investigate the mobility of poly(A)$^+$ RNA throughout the nucleus [101]. 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 [37]. It was also established via atomic force microscopy and transfection
38 experiments that pDNA labeled with quantum dots via PNAs was still functional [103]. 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 [104]. 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 [105].

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 [106, 107]. 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) [109, 110]. 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)

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

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

Binary probes

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

Although this method in theory offers improvements on the high signal background signal of linear oligo probes by employing a light-up principle and offer less chance of non-specific binding by using two independent oligos, some drawbacks should be noted. First of all, the complexity of the design of the probes is even higher than for single linear oligos or MBs[126]. Secondly, the signal of the acceptor fluorophore might suffer from the noise of the cellular autofluorescence. Additionally, the donor and acceptor fluorophores should be chosen in order 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[123]. 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[127], the
6 creation of a lanthanide chelate (see Table 1)[128, 129] and quenched autoligation[130-132] which was
7 used for live-cell imaging of bacterial strains[133] or to visualize mRNA in living cells[134].

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[135]. 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[136]. 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[137]
28 and in intracellular localization[138], due to their uptake in living cells[139]. Vaijayanthi 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[141, 142]. Furthermore FRET was observed when combining two polyamide conjugates with
35 either Cy3® or Cy5®, opening the door to the study of DNA-protein interactions by the gain or
36 loss of the FRET signal[143].

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[144], 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.

Nucleic acid-binding proteins

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

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

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

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

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

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

Table 2 Advantages and disadvantages of the different labeling methods

Labeling method	Pros	Cons	Ideal for:	Available functional groups	Key ref.
Intercalating dyes	+ Easy to use kits available + Fast + Large scale reactions + Light-up principle	- Off-target interactions - Negative effects on NA conformation - Low efficiency dyes	Counterstaining Quantification of cellular uptake	Possibilities all over the visual spectrum (e.g. YOYO-1, TOTO-3)	18 21 9
Covalent attachment	+ Easy to use kits available + Fast + Large scale reactions + Strong covalent attachment	- Negative effects on transfection at high labeling density	Polynucleotide tracking Random labeling	Label-IT: DIG, biotin, DNP, Cy®3, fluorescein, Cy®5, TM-Rhodamine, CX-Rhodamine FastTag: thiol-reactive reagents ULYSIS: Alexa Fluor®488-546-594-647	12 36 13 14
Modified nucleotides	+ Kits available + Broad range of possible methods + End labeling possible	- Prior knowledge of labeling mechanism - Labor intensive - Expensive modified nucleotides	End labeling Random labeling	NHS ester + primary amine Maleimide + thiol Azide + Alkyne Biotin + streptavidin Intrinsically fluorescent labeled nucleotides (see Table 1)	50 52 55
Enzymatic labeling	+ 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®488, NHS ester-AlexaFluor®647, NHS ester-biotin)	66 68
Linear oligos	+ 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)	79 81 85
MBs	+ 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®5)	109 111

Binary probes	+ Highly sequence-specific + Strong attachment + In situ labeling + Light-up principle + Versatile + Reduction of background	- No kit available - Intracellular delivery needed - Sequence information on target needed - Custom design for two adjacent oligos - Optimization needed - High initial price of (modified) probe	Intracellular detection of specific polynucleotides	Virtually all commercial quencher-dye pairs available (e.g. TAMRA – 5'-FAM, Quasar 670 - Cy®5, Ru(II) – Cy®5)	123, 126
NA-binding small molecules	+ Sequence-specific + In situ labeling possible + Spontaneous uptake by cells + Versatile	- No kit available - Sequence information on target needed - Not all sequences can be targeted - Extensive knowledge of polyamide conjugate chemistry necessary - Expensive	Intracellular detection of specific polynucleotides	Same options as for modified nucleotides (see Table 1)	
NA-binding proteins	+ Kits available + Sequence-specific + In situ labeling + Cell compartment specific labeling possible	- Extensive knowledge of FP-tagged proteins needed - Adaption of target with recognition sequences to facilitate binding of proteins - Stable or transient expression of FP-tagged protein in cell needed - Labor intensive - Expensive - Optical properties of FP	Intracellular detection of specific polynucleotides in specific cell compartment	All available fluorescent proteins. Mostly (E)GFP	113 150

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 [162, 163].

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 [164-167].

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 [40, 165]. 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 [168]. By simultaneously labeling different types of endosomal vesicles, the specific type of endosomes from which complexes are preferentially released has been recently identified [169]. Apart from single color-labeling, both the carrier and nucleic acids can be labeled with spectrally separated fluorophores to obtain dual-colored complexes [170]. 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 [104]. 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 [171]. 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 [172]. 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 [173-175]. 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 [176]. Alkyne-bearing nucleotides were for example used to visualize DNA and RNA synthesis *in vivo* by stimulated Raman spectroscopy [177]. 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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