LNA-enhanced DNA FIT-probes for multicolour RNA imaging†

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The simultaneous imaging of different RNA molecules in homogeneous solution is a challenge and requires optimisation to enable unambiguous staining of intracellular RNA targets. Our approach relies on single dye forced intercalation (FIT) probes, in which a visco-sensitive reporter of the thiazole orange (TO) family serves as a surrogate nucleobase and provides enhancements of fluorescence upon hybridisation. Previous FIT probes spanned the cyan and green emission range. Herein, we report for the first time chromophores for FIT probes that emit in the red range (above 600 nm). Such probes are valuable to overcome cellular auto-fluorescent background and enable multiplexed detection. In order to find suitable chromophores, we developed a submonomer approach that facilitated the rapid analysis of different TO family dyes in varied sequence positions. A carboxymethylated 4,4′-methine linked cyanine, which we named quinoline blue (QB), provided exceptional response characteristics at the 605 nm emission maximum. Exceeding previously reported base surrogates, the emission of the QB nucleotide intensified by up to 195-fold upon binding of complementary RNA. Owing to large extinction coefficients and quantum yields (up to ε = 129.000 L mol⁻¹ cm⁻¹ and Φ = 0.47, respectively) QB-FIT probes enable imaging of intracellular mRNA. A mixture of BO-, TO- and QB-containing FIT probes allowed the simultaneous detection of three different RNA targets in homogenous solution. TO- and QB-FIT probes were used to localize oskar mRNA and other polyadenylated mRNA molecules in developing oocytes from Drosophila melanogaster by means of wash-free fluorescent in situ hybridisation and super resolution microscopy (STED).

Introduction

Fluorophore-labelled oligonucleotide probes that increase fluorescence emission upon hybridisation with a complementary target are indispensable tools for the detection and imaging of RNA sequences in biological specimens. One of the goals is to enable the simultaneous detection of different targets, which requires a careful optimisation of label and probe design in order to achieve strong signalling without crosstalk between different readout channels. Conventional probes such as molecular beacons contain two labels, a chromophore and a quencher, and furnish up to 10³-fold increases of fluorescence upon hybridisation-induced separation of the fluorophore–quencher complex.1,2 Probe designs that rely on quenching by aggregation of two chromophores have shown up to 10⁴-fold increase in fluorescence signalling.3–11 However, substantial optimisation of the distance and the position of two reporters is required. With the exception of nanoparticle based systems,12–15 energy transfer-based probes inevitably cover a larger part of the emission spectrum which limits applications in multicolour RNA imaging. Furthermore, background might be significantly increased in complex media such as cells and lysates due to unspecific binding and reduced dye–dye interaction.3 We inferred that a set of singly labelled probes with chromophores in distinct readout channels would be ideal for RNA multiplexing.

Our work relies on quencher-free forced intercalation (FIT)-probes (I, Fig. 1A), in which a single asymmetric cyanine dye of the thiazole orange family serves as an environmentally sensitive nucleobase surrogate.16–19 Hybridisation with a complementary target (I → II) forces the dye to intercalate between predetermined base pairs. The resulting viscosity increase restricts torsions around the methine bridge and prolongs the lifetime of the excited state.20 Previous studies revealed the utility of thiazole orange in real-time PCR,21 wash-free RNA-FISH22 and RNA imaging in living cells.21–28 The combined use of TO and the cyan-emitting BO-reporter allowed the simultaneous detection of two highly expressed viral RNA targets in living cells.23 Yet, the cellular auto fluorescence,
caused, amongst others, by riboflavins, pyridoxine and NAD calls for new, red emissive chromophores.\textsuperscript{30,31}

Herein, we present a method for rapid screening of new chromophores in various FIT probe sequences. By using this method, we discovered quinoline blue (QB) as a notably responsive chromophore in FIT-DNA, affording up to 152-fold enhancement of fluorescence upon hybridization with RNA. While studying the influence of next neighbours, we noticed that the incorporation of a “Locked Nucleic Acid” (LNA) adjacent to the dye nucleotide further improved the fluorescence properties. The combined use of BO, TO and QB nucleotides allowed the simultaneous detection of three different RNA strands without crosstalk. Moreover, the newly developed QB-reporter proved useful in dual colour imaging of total mRNA and oskar mRNA in oocytes of Drosophila melanogaster.

**Results**

The design of dye nucleotides that provide enhancements of fluorescence upon hybridisation and emit in the red spectral range is challenging.\textsuperscript{32–34} Red emitters typically are larger than blue or green emitters and it has to be considered that large dye nucleotides can perturb duplex formation.\textsuperscript{35} Furthermore, in order to be useful in wash-free nucleic acid imaging fluorescence turn-on probes should combine a high fold increase of emission with high brightness, and this property should prevail in different sequence contexts.

In our previous work on DNA FIT probes we relied on preformed dye nucleotides comprised of TO or JO (oxazolo pyridine) dyes and an open-chain backbone analogue based on serinol.\textsuperscript{9,35} To facilitate the screening of different dyes, we developed a universal, N-trifluoroacetyl-protected serinol phosphoramidite building block (1) that simplifies the access of FIT probes by post-DNA-synthetic incorporation of chromophores by activated ester coupling (Fig. 1 and 2A). In the search for red emitting FIT probes we explored TR (thiazole red, far red emission)\textsuperscript{36} as an extended version of TO. Furthermore, we included the 2,4', and 4,4'-methine-linked quinolinium-quinolines QV (quinoline violet) and QB (quinoline blue, names according to appearance in solution) owing to previous reports about viscosity-dependent radiation-less excited state depletions.\textsuperscript{37–40} For comparison, we also investigated the known BO (cyan emission) and TO (green emission) nucleotides.\textsuperscript{19}

**Synthesis**

The chromophores QV, QB and TR were synthesized according to published procedures with slight modification to obtain carboxymethyl linkages, which are crucial for the use in DNA FIT-probes (see Scheme S1†).\textsuperscript{41} The universal Ser(TFA) phosphoramidite 1 is shelf-stable and was accessed in a 5-step synthesis starting from commercially available L-serine methylester (Scheme S1†). Each chromophore was placed at 15 different positions within a 27mer sequence directed against influenza H1N1-neuraminidase-mRNA (Table 1).\textsuperscript{42} TO- and BO-labelling was performed by means of the previously reported Ser(BO) and Ser(TO) phosphoramidites.\textsuperscript{19} For the introduction of the new chromophores, oligonucleotides carrying Ser(TFA) as a universal precursor were assembled by automated DNA-synthesis (Fig. 2B) and purified by reversed phase (RP)-HPLC. The TFA group was removed under standard cleavage conditions. Dye-NHS-esters were prepared in DMF by using the chromophore, pyridinium p-toluene sulfonate (PPTS, for
enhanced solubility of the chromophores), diisopropylcarbodiimide (DIC) and N-hydroxysuccinimide (NHS) and reacted with the Ser(NH$_2$) oligonucleotide (2) for 1 h at 30 °C (Fig. 2C). Precipitation by isopropanol and subsequent purification by RP-HPLC provided pure FIT-probes 3 in an overall 30–60% yield (Fig. 2D).  

### Fluorescence properties

The fluorescence emission of the colour expanded FIT probes was measured before ($I_0$) and after hybridisation ($I$) with complementary RNA target. The probe concentration was assessed by UV-absorbance measurements and fluorescence emission was corrected accordingly (Table 1 and Fig. 3). Previous experiments had revealed that the introduction of a locked nucleic acid (LNA) monomer adjacent to the dye nucleotide afforded particularly bright DNA FIT probes. Yet for simplicity we excluded LNA in the initial screen. The known Ser(TO) nucleotide was walked through the 15 positions of the neuraminidase mRNA probe neu. As shown previously, the fluorescence enhancement for TO ranges from 1.6 to 12-fold with 4 probes that show $I/I_0 \approx 10$. In contrast, the BO-nucleotide provided lower response factors (0.6–5.6-fold), but high overall intensity. Of note, QV-labelled probes furnished up to 50-fold fluorescence enhancement (ranging from 1.4 to 50-fold), but the absolute intensity of QV emission was rather low (~1/3 of intensity of TO emission, see Table 1 and Fig. 3C). Though low brightness is not an issue for *in vitro* measurements, we expect limitations in cellular RNA imaging. A remarkably strong fluorescence intensification upon hybridisation (152-fold, neu-QB-m, Fig. 3D) was obtained with the QB-labelled FIT probes. The minimal fluorescence enhancement obtained in this study was still 6.3-fold. In contrast to QV, the intensity of QB was comparable to TO, suggesting a usefulness in RNA imaging studies (*vide infra*). The TR-containing oligonucleotides showed very bright fluorescence emission. However, the emission was strong in both single stranded and target-bound states and so the TR probes remained virtually non-responsive towards hybridisation to complementary RNA.

| Sequence | X = Ser(BO) | X = Ser(TO) | X = Ser(QV) | X = Ser(QB) | X = Ser(TR) |
|----------|-------------|-------------|-------------|-------------|-------------|
| a | GGTTTCAGTTATTATGCCGTXGTATTT | 18 39 2.2 | 4.3 29 6.7 | 0.5 9 17.7 | 0.7 20 28.3 | 94 86 0.9 |
| b | GGTTTCAXTATTATGCCGTXGTATTT | 19 66 3.8 | 4.8 48 5.6 | 1.1 11 9.9 | 1.1 28 25.5 | 101 98 1.0 |
| c | GGTTTCAGXTATTATGCCGTXGTATTT | 20 89 2.8 | 5.8 68 12.2 | 0.9 11 12.3 | 1.6 24 15.4 | 105 74 0.7 |
| d | GGTTTCAGXTATTATGCCGTXGTATTT | 21 22 1.1 | 4.9 18 3.7 | 0.5 4 6.9 | 0.9 27 30.3 | 98 82 0.8 |
| e | GGTTTCAGXTATTATGCCGTXGTATTT | 22 46 3.0 | 6.2 66 10.6 | 0.5 20 39.8 | 0.9 58 63.6 | 92 111 1.2 |
| f | GGTTTCAGTXATTATGCCGTXGTATTT | 23 41 1.1 | 3.8 19 5.0 | 0.9 4 4.6 | 1.1 24 22.6 | 114 91 0.8 |
| g | GGTTTCAGTXATTATGCCGTXGTATTT | 24 20 0.6 | 9.7 16 1.6 | 1.0 4 3.7 | 1.4 19 13.7 | 125 86 0.7 |
| h | GGTTTCAGTXATTATGCCGTXGTATTT | 25 31 1.3 | 8.6 54 6.2 | 1.0 20 20.9 | 0.9 38 42.3 | 117 128 1.1 |
| i | GGTTTCAGTXATTATGCCGTXGTATTT | 26 33 1.8 | 0.6 7.6 | 11 1.4 1.3 | 2 1.4 1.4 9 6.3 102 72 0.7 |
| j | GGTTTCAGTXATTATGCCGTXGTATTT | 27 32 1.4 | 13.5 42 3.2 | 1.6 18 11.1 | 3.0 30 10.1 | 110 99 0.9 |
| k | GGTTTCAGTXATTATGCCGTXGTATTT | 28 17 9.9 | 5.6 8.7 42 | 4.8 1.3 10 | 8.0 1.6 21 13.6 | 98 56 0.6 |
| l | GGTTTCAGTXATTATGCCGTXGTATTT | 29 23 3.9 | 7.6 23 6.6 | 0.4 4 10.7 | 0.7 29 43.7 | 83 54 0.7 |
| m | GGTTTCAGTXATTATGCCGTXGTATTT | 30 36 5.0 | 1.4 4.2 41 | 9.7 0.4 20 | 49.8 0.5 74 152 | 81 142 1.8 |
| n | GGTTTCAGTXATTATGCCGTXGTATTT | 31 17 6.6 | 3.8 4.4 52 | 12.0 0.5 7 15.3 | 0.8 25 31.5 66 104 1.0 |
| o | GGTTTCAGTXATTATGCCGTXGTATTT | 32 25 2.2 | 0.9 3.5 11 | 3.3 0.5 2 | 4.7 1.2 43 35.4 117 79 0.7 |
| Mean values | 25 49 2.2 | 6 36 6.2 | 1 10 14.5 | 1 31 35.6 | 100 91 0.9 |
Figure 3 Absorbance and emission spectra of the most responsive probes carrying (A) BO, (B) TO, (C) QV, (D) QB and (E) TR. Spectra are corrected for probe concentration. Conditions: 0.5 µM probe and 5 eq. RNA target, when added, in PBS at 37 °C. BO: λex = 455 nm; TO: λex = 485 nm; QV: λex = 530 nm; QB: λex = 560 nm; λem = 610 nm; slitex = 5 nm, slitem = 5 nm. Analytical data, absorbance and emission spectra of all probes are provided in the ESI (Table S1–S6 and Fig. S1–S5†).

Multi-colour readout

The ability to simultaneously detect multiple RNA targets is envisioned to facilitate RNA imaging experiments, particularly when different RNAs of interest or splicing variants are to be detected. The fluorescence spectra of BO, TO and QB can be resolved and cover the cyan (BO), green (TO) and red (QB) range of the visible light spectrum. Thus, multi-colour readout by FIT probes can be carried out by standard fluorescence microscopy equipment. The Ser(BO)-, Ser(TO)- and Ser(QB)-nucleotides were included in three gapmer FIT probes targeting three different RNA sequences. The second LNA building block in the BO-probe was included to improve the target affinity of the rather short oligonucleotide. The three readout channels were simultaneously detected in a kinetic experiment (Fig. 5). The probes were sequentially added to buffer (BO: 3 min, TO: 7 min, QB: 10 min) followed by sequential addition of each complementary RNA (BO-RNA: 13 min, TO-RNA: 17 min, QB-RNA: 20 min). The ascending order from blue to red emission range was chosen to assess potential cross-excitation. In spite of the combination of probes and the excess of targets, neither cross-excitation nor energy transfer of the different readout channels was observed. The addition of probe resulted immediately in a stable signal, indicating the absence of folding or aggregation. Furthermore, the probes responded rapidly to the addition of target RNA and within seconds afforded 20–69-fold enhancements of fluorescence, while non-complementary RNA produced only a marginal increase in fluorescence, if at all. The near instantaneous response points to one of the advantages
**Table 2** Fluorescence properties of TO-labelled FIT probes directed against vasa mRNA. Conditions: see Table 1

| Name             | Sequence\(a\), \(X = \text{Ser(QB)}\) | \(\Phi_0\) | \(\Phi\) | \(\Phi/\Phi_0\) | \(I/I_0\) |
|------------------|------------------------------------------|-----------|--------|--------------|----------|
| vasa-QB-1        | UCUAUUUUCXTCAUUUCA                       | 0.007     | 0.18   | 25.7         | 65.0     |
| vasa-QB-1LNA     | UCUAUUUUCXT1CAUUUCA                      | 0.004     | 0.45   | 112.5        | 195.1    |
| vasa-1-RNA       | UGAAAGAUGTAGAAATAGA                      |           |        |              |          |
| vasa-QB-2        | UCCAUUUUCXTTAAUUUCU                      | 0.004     | 0.31   | 77.5         | 52.2     |
| vasa-QB-2LNA     | UCCAUUUCXT1TAUUUUC                       | 0.009     | 0.47   | 52.2         | 69.6     |
| vasa-2-RNA       | AGAAAUAAUGAAUUGAGA                       |           |        |              |          |
| vasa-QB-3        | GGGCAGCGUGTXTTCUGG                       | 0.006     | 0.27   | 45.0         | 42.8     |
| vasa-QB-3LNA     | GGGCAGCGUGTXT1TCUGG                      | 0.007     | 0.47   | 67.1         | 125.2    |
| vasa-3-RNA       | CAGGAAAAACGGGGGCC                        |           |        |              |          |
| vasa-QB-4        | GGAACUAUCXTGUGGUCG                       | 0.020     | 0.24   | 12.0         | 14.0     |
| vasa-QB-4LNA     | GGAACUAUCXT1GUUGGC                       | 0.012     | 0.45   | 37.5         | 68.9     |
| vasa-4-RNA       | GCCCCAACAUGUUC                           |           |        |              |          |

\(a\) LNA nucleotides are printed in boldface and indicated by subscript “\(L\)”; \(2’\)-O-Me nucleotides are underlined.

provided by non-structured probes not shared by molecular beacon type probes.48 Owing to the LNA enhancement, the probes exhibited fluorescence of comparable intensities, which will facilitate multiple colour readouts in cells. The low background signal and the high brightness of FIT probes in their target-bound state allowed the detection of complementary target at low \(\mu \text{M}\) concentrations by the naked eye upon excitation with a handheld UV-lamp (see Fig. S7†).

**Wash-free FISH**

To assess the usefulness of QB-labelled probes in multicolour RNA imaging, we set out to analyse the localisation of oskar mRNA and total mRNA in fixed Drosophila egg-chambers by wash-free FISH.28 The egg-chamber consists of an oocyte connected to its 15 sibling nurse cells and is encapsulated by a single layer of follicular epithelial cells. To demonstrate that multiplexing works in situ, we used an 18mer LNA-modified oligo-T-probe (TTTTTTTTT-Ser(QB)-TTTTTTTTT) \(\text{dT(18)-QB}\) targeted against the poly-A tail of mRNA, which should be distributed all over the specimen, and a mixture of five LNA-modified TO-labeled probes targeted against oskar mRNA (osk-TO-LNA1-5), which should localize at the posterior pole of the oocyte.28 In cuvette experiments, the oligo-dT-probe provided 89-fold enhancement of QB emission (605 nm) upon hybridisation with complementary RNA (Fig. S8†). Previously, we have shown that the oskar mRNA specific TO-FIT probes afforded response factors \(I/I_0 = 6–13\) (Table S9†).28

The oskar mRNA is produced in the nurse cells and transported to the posterior pole of the oocytes. The fluorescence microscopy analysis (Fig. 6A and A’) confirmed that the TO
signal was mainly detected at the posterior, where oskar localizes. In contrast, imaging QB showed a more uniform distribution of the poly-dT probe, as expected. Interestingly, however, we observed a mild accumulation of QB signals at the posterior pole (Fig. 6A’, yellow arrow), where not only oskar but many other mRNAs were shown to enrich. Accordingly, when we performed super-resolution imaging on the posterior pole of stage 10 oocytes using stimulated emission depletion (STED) microscopy, we found that many but not all dT_{18}-QB positive particles are positive for oskar (Fig. 6D, see also Fig. S10A and B†). As a control, staining was attempted with the vasa-QB-2LNA probe (Fig. S9†). This probe has no target in Drosophila melanogaster and therefore only showed dim background signals.

We also found accumulation of the oligo-dT-QB probe around the nurse cell nuclei (Fig. 6A”, white arrows). These cells – unlike the oocyte – are transcriptionally highly active, yet a large fraction of the synthesized mRNA species enrich within the oocyte. We think that this accumulation of poly-A(+) mRNA is oskar mRNA itself, and similar to what we observed at the oocyte posterior pole, a fraction of dT_{18}-QB particles co-localized with oskar (Fig. S9C and D†). Interestingly, the dT_{18}-QB-stained particles within the perinuclear region are relatively large and appear to be regularly spaced (Fig. S10C and D†). Another previously undetected enrichment of dT probe signal was observed within the meiotic and thus translationally silent nucleus of the oocyte (Fig. 6B, arrowhead), where we observed speckles of unknown nature (Fig. S9, ESI movie†). Of note, these speckles also appeared when staining was performed with Texas Red labelled dT_{19}-probes, though contrast after normalization was significantly lower than for the dT_{18}-QB probe (Fig. 6C).

**Discussion**

The aim of this work was to expand the colour repertoire of DNA-based FIT-probes in order to enable multicolour RNA imaging. To streamline the screening for suitable dyes we established a method for the synthesis of DNA FIT probes based on a universal N'-Tfa-protected serinol phosphoramidite, which allowed the post-synthetic coupling of dye-NHS-esters onto oligonucleotides. Similar methods based on NHS-ester coupling are routinely used for terminal labelling of molecular beacons or for modification of nucleobases in ECHO-probes. The coupling succeeded on small scale and as a result the

![Fig. 6](image)

**Fig. 6** Wash-free FISH of fixed Drosophila egg chambers. (A–A”) Egg chambers comprising the translationally active nurse cells interconnected to the meiotic oocyte, surrounded by a layer of somatic follicular epithelium were co-labelled with a mixture of (A’) five TO-labelled oskar probes (osk-TOLNA1-5, 0.1 μM each) and (A”) dT_{18} (0.5 μM) QB. (B and C) Normalized images of egg chambers labelled with (B) dT_{18}-QB, (C) TexasRed-labelled dT_{19} (0.5 μM). Scale bars represent 50 microns (A–C). Normalization was done against the background signal measured within the oocyte nuclei (Fig. S10B† cyan dashed circle). White arrows indicate accumulated RNA around the nurse cell nuclei; yellow arrows indicate accumulation of oskar mRNA at the posterior pole; white arrowheads indicate speckles in the oocyte nucleus. (D) Super-resolution image (STED) of the oocyte posterior region of a stage 10 egg chamber, where oskar mRNA is enriched. The inset is an enlarged version of the image area within the dashed box. Scale bar represents five microns; scale bar in the inset represents one micron. Wash-free FISH of fixed Drosophila egg chambers.
method greatly facilitated the study of different chromophores in equal sequence contexts.

We identified a 4,4′-linked cyanine as a particularly responsive chromophore in FIT-probes which provided 10^2-fold fluorescence enhancement upon hybridization with RNA target. The dye has been the subject of intensive photophysical studies in which the barrier-less isomerization was scrutinized in fine detail.\textsuperscript{18,40,52–56} The viscosity-dependence of excited state deactivation by photoisomerisation is a property common to many monomethine chromophores of the thiazole orange family of DNA intercalators. To illustrate this relation we coined the term quinoline blue (describes structural detail and colour in solution). Like many TO family members QB has low fluorescence in free form,\textsuperscript{26} yet experiences dramatic enhancements of fluorescence upon binding to DNA. This has been noticed previously when QB was attached to terminal and internal positions of oligonucleotides by means of 8–12 atom-long linkers.\textsuperscript{19} However, the fluorescence of the previously explored DNA-conjugates was high regardless of the hybridization state. This illustrates a key feature of FIT probes. The use of a very short carboxymethyl linker presumably provides steric hindrance and perturbs dye–nucleotide interactions in cis and in trans. As a result, FIT probes remain dark unless hybridization with a target molecule enforces the intercalation between pre-determined base pairs. The accompanying viscosity increase hinders torsional motions around the methine bridge so that fluorescence can occur. This effect is augmented when LNA is placed adjacent to FIT-reporters. As previously reported for TO, a single LNA adjacent to the QB reporter resulted in significantly increased quantum yield (DNA(mean) $\Phi$ = 0.25; LNA(mean) $\Phi$ = 0.46) and brightness (see also Table S8).\textsuperscript{26,57} Similar improvements of the photophysical properties of dyes on introduction of flanking LNA monomers have recently been reported by Hrdlicka.\textsuperscript{27} Moreover, the emission in the red range (575–700 nm) is orthogonal to BO and TO and allows RNA multiplexing without any false positive signalling by the presence of other probes or non-complementary RNA target.

The fluorescence microscopic analysis of developing oocytes from Drosophila melanogaster revealed the localization of two different mRNA targets. The poly-A-tail specific QB-FIT probes revealed a quite uniform distribution of total mRNA in the specimen with enrichment at the perinuclear region of the transcriptionally active nuclei of the nurse cells. The discovery of speckles in the transcriptionally silent nucleus of the oocyte observed with the QB-FIT probes and, at lower overall contrast, with Texas Red-dT\textsubscript{19} probes is noteworthy. The combined use of the two espectively resolvable probes confirmed the enrichment of mRNA at the posterior pole from mid-oogenesis onwards, including oskar mRNA and other poly-A tail-containing mRNA molecules.

Conclusions

We developed a method for the simplified screening of DNA FIT probes that respond to hybridisation by showing enhancement of fluorescence in the red spectral range. The synthesis method relies upon an N-trifluoroacetyl-protected serinol phosphoramidite, which is introduced during automated DNA synthesis and allows the solution-phase coupling of thiazole orange family cyanine dyes. According to the design criteria for forced intercalation probes, the obtained oligonucleotide conjugates contain a carboxymethyl-linked cyanine dye rather than a nucleobase at a sequence-internal position. The comparison of 5 different dyes in 15 different sequence positions revealed a quinoline based 4,4′-cyanine (QB) as a particularly suitable dye. Among the single-labelled hybridisation probes reported in the literature, QB-FIT probes provide the highest fluorescence enhancement (up to 195-fold) upon hybridisation with complementary RNA. The red emission ($\lambda_{em}(max)$ $\approx$ 605 nm) is orthogonal to the previously reported BO- and TO-FIT probes and allows their combined use in multiplexing experiments. The brightness of QB can be significantly improved by introducing an adjacent LNA-nucleotide. The usefulness of the QB dye in FIT probes was demonstrated in dual colour RNA imaging in developing oocytes from Drosophila melanogaster. The combined use of five different TO-FIT probes targeted against oskar mRNA and a QB-FIT probe designed for recognition of the mRNA poly-A tail revealed the different localization of the two targets when used in wash-free fluorescent in situ hybridisation, and exposed hitherto unknown signals from fluorescence-labelled oligo-dT\textsubscript{19} probes in the meniscus of the oocyte. In this investigation we also showed for the first time super-resolution microscopy with FIT probes by stimulated emission depletion (STED). Based on the robustness and brightness of fluorescence signalling by QB-FIT probes and the documented usefulness of TO-FIT probes we are convinced that FIT probes will probe useful in many other multiplexed RNA imaging endeavours.

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