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FISH and immunocytochemistry: towards visualising single target molecules in living cells

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Knowledge of how molecules interact in space and time is crucial for understanding cellular processes. A host of novel techniques have been developed for the visualisation of single target molecules in living cells, many based on fluorescence *in situ* hybridisation (FISH) or immunocytochemistry (IC). To extend the applicability of FISH to living cells, special backbone-modified probes and specific conformations (molecular beacons) have been designed. In the case of IC, conventional immunoreagents have been fine-tuned with respect to size and affinity or replaced with new protein scaffolds based on ankyrin repeat proteins. Other key advances include the use of proximity ligation to confirm vicinity binding and the use of quantum dots, which have proven potential for cellular labelling.

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Abbreviations

FISH fluorescence *in situ* hybridization
FRET fluorescence resonance energy transfer
IC immunocytochemistry
PNA peptide nucleic acid
QD quantum dot

Introduction

A substantial part of current research in biology is devoted to the understanding of the molecular mechanisms that underlie the function and activity of cells and tissues. In medicine this knowledge is used to improve diagnosis and to find target molecules for novel therapies. Biochemical analysis of cell homogenates provides information about the type, amount and structure of biomolecules present under various biological circumstances. However, an essential step is to link the molecular information to the behaviour of individual cells, as knowledge about

molecular interactions in space and time is crucial to understand the regulatory machinery.

This demand has led to improved and novel microscopic techniques, as well as cytochemical staining methods, aiming to provide better resolution, sensitivity, specificity and versatility. Main concepts for the specific staining of cellular molecules are fluorescence *in situ* hybridisation (FISH), which uses fluorescent nucleic acids to stain complementary sequences by hybridisation, and immunocytochemistry (IC), which utilises the biological affinity between antigens and antibodies, or variances thereof.

This review summarises recent methodological advances in FISH and IC and in the inherent fluorescent reporter technology used.

FISH: on the move from *in situ* to *in vivo*

The technology for detection of nucleic acid sequences in fixed biological structures by FISH is well-established [1] and now appears to be reaching its methodological limits. Here we will briefly discuss single-base mismatch DNA detection *in situ* as well as advances in RNA detection. Most of the recent FISH developments have been directed towards living cells, here referred to as '*in vivo*' hybridisation.

Padlock probing for single-base mismatch DNA detection by FISH

The sensitivity of conventional DNA FISH is well established. The smallest nucleic acid sequence detectable by FISH is a single-copy fragment of a few hundred nucleotides. Target loss and limited accessibility are likely to be the limiting factors in this method. However, the efficiency of detecting short sub-kilobase target sequences is too low for routine application and essentially precludes reaching the holy grail of FISH: *in situ* single-base mismatch detection at the single-copy gene level. Convincing *in situ* mismatch detection has been limited to repetitive targets only [2].

A padlock probe is a linear oligonucleotide with 5' and 3' sequences that are complementary to the target sequence. In between them is a linker of irrelevant sequence that carries immunologically detectable residues such as biotin and digoxigenin. Following hybridisation of a padlock probe to its perfectly complementary sequence, a ligase reaction covalently links the 3'- and 5'-ends of the padlock probe, thereby locking it onto its target strand by circularisation. If the padlock hybridises

to a single base-mismatching sequence, the padlock probe will not be ligated and will dissociate during subsequent stringent washes. With its mismatch-sensitive ligation step and its high-stringency washing potential, padlock probing has been used successfully in FISH for repeat-sequence variant detection [3]. In combination with *in situ* rolling circle amplification, padlock-based FISH has recently also been used successfully for the localised detection of point mutations in mitochondrial DNA (M Nilsson, personal communication; see Update).

Pushing RNA FISH to the limits

Singer and co-workers [4] improved the sensitivity of RNA FISH by using a combinatorial approach and three to five oligonucleotides per transcript, with each probe having multiple fluorophores of a given kind. In conjunction with advanced computational fluorescence microscopy they managed to create transcription profiles for up to eleven genes in the nuclei of *in vitro* cultured cells. Recently, Kosman *et al.* [5] performed sophisticated RNA FISH in *Drosophila melanogaster* embryos to visualise six different transcripts simultaneously and exploited ribo-probes and tyramide signal amplification methods developed in the 1990s.

General considerations for *in vivo* hybridisation

In vivo hybridisation depends upon the efficient delivery of fluorescently labelled probe molecules into living cells. Microinjection, streptolysin O, scrape-loading, peptide-mediated membrane transfer and electroporation have all been applied for *in vivo* hybridisation studies, each method having its own pros and cons [6].

Because cells are full of nucleases, degradation of nucleic acid probes is a serious problem for *in vivo* hybridisation studies. Peptide nucleic acid (PNA) probes and 2'-O-methyl RNA are examples of modified backbones that serve the purpose of nuclease-resistance in the '*in vivo*' hybridisation studies published so far. One of the beauties of FISH on fixed cells is that hybridisation stringency conditions can be chosen at will. Also, excess non-bound probe can be washed away to improve the distinction between the signal and background noise. This is not possible for *in vivo* hybridisation, where either a fluorogenic approach needs to be taken or a limiting amount of labelled probe is to be delivered in the cell so that the target mRNA is at, or close to, saturation. Obviously, in the latter case non-specific interactions will co-exist in the cell, and because stringency manipulation is not possible the probes intrinsically need a strong preference for their intended targets. Fortunately, backbone modifications that result in resistance to nucleolytic breakdown also increase the specificity of hybridisation by increasing the affinity between the backbone-modified probe and the target. Using a fluorescently labelled 2'-O-methyl RNA probe complementary to the poly(A) tail of mRNAs, Molenaar *et al.* [7]

showed that shortly after injection of the probe a nuclear labelling pattern specific for poly(A) RNA was obtained. By contrast, when a DNA probe complementary to the poly(A) tail of mRNA was injected, no specific staining pattern was observed.

Molecular beacons and '*in vivo*' mRNA hybridisation

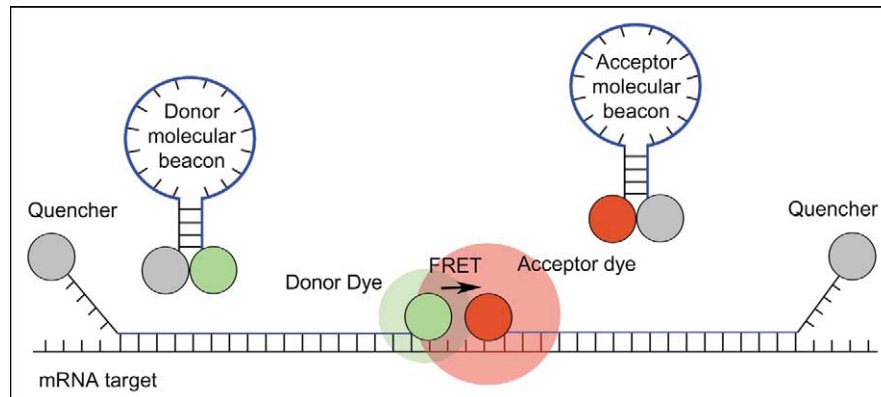
A molecular beacon is a hairpin structure consisting of a probe sequence (loop) and complementary 3' and 5' ends (stem) that carry a fluorophore and a quencher. In the stem-loop configuration, there exists virtually complete quenching by fluorescence resonance energy transfer (FRET), whereas upon hybridisation to its target the beacon opens up and becomes fluorescent [8]. A molecular beacon is not only a fluorogenic probe but also possesses the greater power of discriminating single-base mismatching sequences from their linear, non-hairpin counterparts [9].

However, molecular beacons tend to open-up and start to fluoresce when brought inside living cells, as they interact with cellular constituents that are not targets of the molecular beacons [10]. To overcome this problem, a FRET strategy was developed based on fluorescent labels being brought into close proximity through the hybridization of two hairpin-type oligonucleotides, labelled at the 3' and 5' end with a FRET donor and acceptor, respectively, to two neighbouring target sequences (Figure 1) [11*,12]. Thus, the specificity of the signal is primarily a consequence of two independent hybridisation events that bring two spectrally overlapping fluorophores into close and fixed proximity on the target molecules. This creates a very high FRET potential amidst a large pool of non-FRET fluorescence. The chances for non-specifically bound, fluorescing molecular beacons to exhibit detectable FRET are small. Alternatively, Bratu *et al.* [11*] suggested that one could monitor the non-specific opening of beacons by co-injecting a beacon with an irrelevant sequence, and then map the localisation of the specific hybridisation through ratio-imaging.

Peptide nucleic acids and '*in vivo*' DNA hybridisation

The imaging of specific DNA sequences in living cells is an even greater challenge than detecting RNA sequences owing to the double-stranded nature of DNA. Because denaturing conditions to generate single-stranded DNA cannot be applied to living cells, probes need to be designed that have strand displacement properties. Such properties have been described for PNA probes, although these probes have so far only been successful in detecting telomeric sequences in living cells. Using a Cy3-labelled telomere-specific PNA probe that was injected in U2OS osteosarcoma cells, it proved possible to efficiently label the telomeres and to track their movements in space and time using confocal imaging microscopy [13] (Figure 2). Attempts to hybridise PNA probes to repetitive centro-

Figure 1



Schematic illustration of the dual molecular beacon FRET approach for *in vivo* detection of specific mRNA (taken from [37]). The 'green' FRET donor fluorescent dye at the 3' end of the donor molecular beacon will only be positioned in close proximity of the 'red' FRET acceptor fluorescent dye at the 5' end of the acceptor molecular beacon by two independent hybridisation events on the specific mRNA molecule. Thus a FRET signal will only emanate from sites where both beacons are bound and not from sites with non-specifically bound (that is singly bound) acceptor or donor molecular beacons.

meric DNA regions in living cells have not been successful so far. Thus, it cannot be excluded that it is the specific structural properties of telomeres that enable PNA probes to have access to them.

Immunocytochemistry: alternatives to conventional antibodies and novel reporters

The great majority of research reports that employ techniques for microscopically localising specific proteins use the well-established principles of IC. In recent years several alternatives to conventional antibodies have been described, as has the use of proximity ligation for high specificity detection on the basis of dual recognition. Finally, a new reporting technology using quantum dots (QDs) is about to come of age.

Camelidae immunoglobulins

In 1993, it was found that *Camelidae* naturally produce single heavy-chain antibodies (referred to as V_HHs) of only 14 kDa, with high specificity and affinity [14]. They are easily produced by phage display techniques and have been used for immunoaffinity purification [15]. Recently they have also been used for immunocytochemical purposes [16]. Their small size, and the possibility to express them as a fusion protein with green fluorescent protein, makes them potential candidates for '*in vivo*' IC. For example, a camelid antibody fragment was shown to inhibit the *in vitro* aggregation of amyloid fibrils by human lysozyme [17].

Ankyrin repeats

A different approach is the design of alternative protein scaffolds, which relies upon the modularity of ankyrin repeat proteins. Combinatorial libraries of consensus-designed ankyrin repeat proteins with randomised poten-

tial interaction surfaces have been generated, from which binding molecules were selected. Designed ankyrin repeat proteins are especially suited for intracellular applications, because of their high stability, absence of disulfide bonds and selectable high affinities, where antibodies are less than ideal [18].

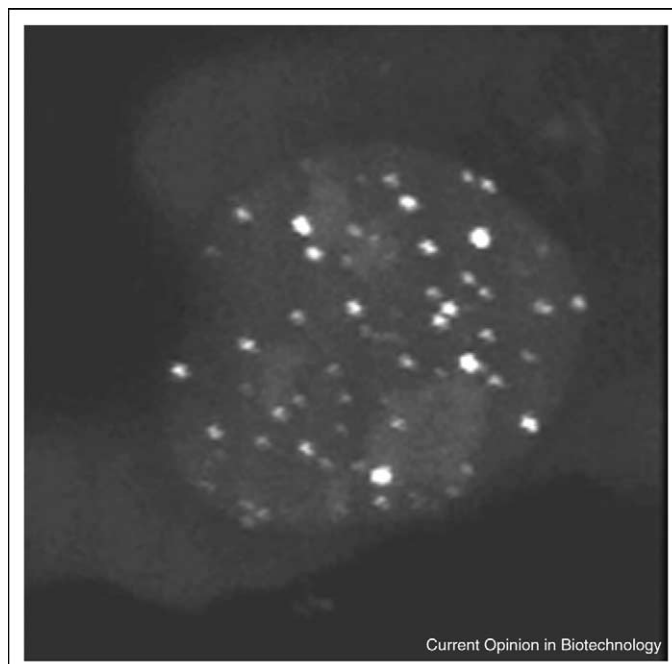
Proximity ligation for *in situ* protein detection

Two approaches have been taken to exploit the 'proximity ligation' of nucleic acids for *in situ* protein detection [19*]. In the first approach, two different aptamers that bind specifically to protein-binding partners are extended with sequences to be ligated through mediation of a common connector sequence. Ligation, and with it the proximity of the binding partners, is then detected through, for example, real-time monitoring of nucleic acid amplification. Alternatively, and to become independent of aptamer selection, different antibodies binding to the target protein or protein dimer of interest are covalently linked to oligonucleotides. Following binding of the antibodies to the (interacting) target molecule(s), ligation and detection of the ligated product are performed [20]. When using padlock probes, the circularised connector oligonucleotide can then be used for localised, *in situ* amplification and detection (U Landegren, personal communication). Successful application of localised rolling circle amplification triggered from modified antibodies in protein array and IC applications has been accomplished [21,22].

New reporter technology

In a recent article it was concluded that colloidal inorganic semiconductor nanocrystals, or QDs, have finally come of age [23]. In two aspects they represent the ideal fluorescence reporter in FISH and IC: they are not subjected to

Figure 2

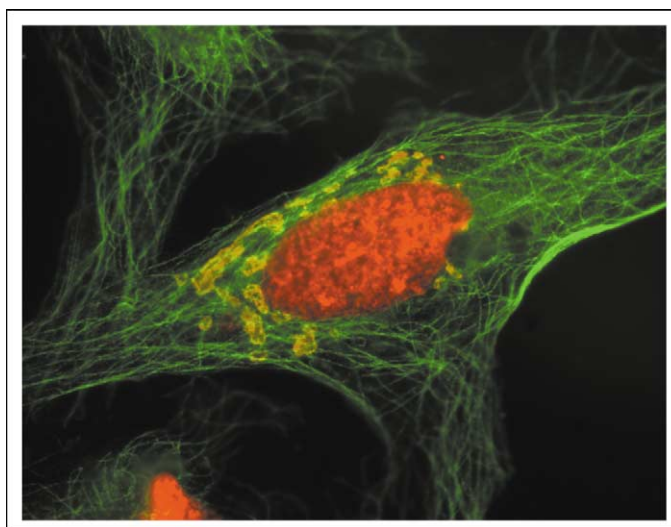


Human osteosarcoma cell nucleus (U2OS cell line) vitally injected with a Cy3-labelled probe specific for chromosomal telomeric repeat sequences. Note that some spots are relatively large suggesting association or clustering of multiple telomeres. For details and examples of telomere dynamics see [13].

photobleaching and QDs of different emission properties (colours) can all be excited with a single wavelength bandwidth. Fundamental problems related to the use of QDs in the physiological milieu, such as the control of photophysical properties in aqueous solution, non-

specific binding and aggregation, have been overcome, and reproducible procedures for synthesis and conjugation recently have been established. The key to success was the use of amphiphilic compounds to coat the surface of the hydrophobic semiconductor particles; examples

Figure 3



HeLa cells immunostained stained with QD conjugates of three different colours: green, microtubules; orange, Golgi; red, nuclei. The illustration is provided by Quantum Dot Corporation, Hayward CA.

include the use of hydrophobically modified polyacrylic acid conjugated to immunoreagents [24], peptides and polyethylene glycol that are co-adsorbed to the surface [25] or encapsulation of the particles in micelle structures [26].

In these forms QDs have been successfully used for immunolabelling of cellular molecules, such as Her2, actin filaments [27] and other cellular targets [24,28], to study diffusion dynamics of glycine receptors [29], and as probe labels for FISH on human metaphase chromosomes [30] (Figure 3). Not hampered by problems related to biocompatibility, QDs are now used for lineage tracking experiments in embryogenesis [26,31] and have a great potential for *in vivo* cancer targeting and imaging [32], particularly when multiphoton imaging is applied [33]. QDs are also well suited for correlative fluorescence and electron microscopy studies [34].

Another particle-based reporter technology consists of microcrystals of upconverting phosphors, which emit visible light upon excitation with infrared light, thereby totally avoiding the unwanted autofluorescence of biological specimens upon shorter wavelength excitation; however, FISH and IC applications are limited to date [35].

Conclusions

One might conclude that, for fixed cells, the future methodological advances of FISH and IC will relate to application-specific fine-tuning of sensitivity and multiplicity, and that the real challenge for the coming years is the application to living cells. *In vivo* applications of 2'-O-methyl RNA probes, PNAs and molecular beacons will mature, and other strategies (or combinations of existing ones) are expected. Whereas current hybridisation technology, in principle, allows for the detection of transcripts in live cells, detection of double-stranded DNA sequences under these conditions is, with the exception of telomere repeats, not yet possible. New principles might be found in the generation of libraries of proteins or small RNA molecules that show defined interactions with DNA sequences, either in the double- or single-stranded form.

The potential to find suitable alternatives to conventional antibodies for *in vivo* application is certainly there. How widely single heavy-chain *Camelidae* antibodies will be used for demonstrating intracellular targets under *in vivo* conditions and whether or not ankyrin repeats offer a good alternative will strongly depend upon the ease with which these proteins can be generated and the participation of the biotech industry. Finally, the conclusive maturation of nanocrystal technology (QDs) is highly welcome, although some crucial issues need further elucidation: are QDs suited for FRET and fluorescence lifetime imaging, and is the same type of information about the association or conformation of target molecules obtained

as with soluble fluorophores? Also, is their physical size (5–20 nm) too large to approach all cellular targets (an immunoglobulin molecule is about 4–5 nm)? Despite these questions, QDs convincingly demonstrate how developments in material physics and chemistry will help us to study molecular processes in live cells, a situation that has just begun and will continue in the future [36].

Update

The reference cited in the main text as M Nilsson, personal communication has now been accepted for publication [38].

Acknowledgements

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