Single-molecule fluorescence in situ hybridization: Quantitative imaging of single RNA molecules

Sunjong Kwon*
Department of Biomedical Engineering, Oregon Health & Science University, Portland, OR 97239, USA

In situ detection of RNAs is becoming increasingly important for analysis of gene expression within and between intact cells in tissues. International genomics efforts are now cataloging patterns of RNA transcription that play roles in cell function, differentiation, and disease formation, and they are demonstrating the importance of coding and noncoding RNA transcripts in these processes. However, these techniques typically provide ensemble averages of transcription across many cells. In situ hybridization-based analysis methods complement these studies by providing information about how expression levels change between cells within normal and diseased tissues, and they provide information about the localization of transcripts within cells, which is important in understanding mechanisms of gene regulation. Multi-color, single-molecule fluorescence in situ hybridization (smFISH) is particularly useful since it enables analysis of several different transcripts simultaneously. Combining smFISH with immunofluorescent protein detection provides additional information about the association between transcription level, cellular localization, and protein expression in individual cells. [BMB Reports 2013; 46(2): 65-72]

INTRODUCTION

In 2012, in a coordinated set of 30 papers, the Encyclopedia of DNA Elements (ENCODE) released initial results of a project, the goal of which was to identify all functional elements in the human genome sequences (1). One of the striking findings was that almost two-thirds of the human genome is transcribed into RNA sequences (2). Many of which do not code for proteins (2). Many of the noncoding transcripts appear to play important regulatory roles and should be considered as important inherited genetic elements. Another striking result was the discovery that almost two-thirds of the human genome is transcribed into noncoding RNA transcripts in more or less intact cells. ISH is particularly powerful since it enables the interrogation of almost the entire transcriptome and genome, including alternately spliced and rearranged forms. Furthermore, fluorescence in situ hybridization (FISH) has been able to identify the total copy number of mRNAs in intact cells and tissues at the single-molecule/single-cell level (3). FISH has even resulted in the detection of nascent mRNAs at the site of transcription (4).

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or mask subcellular localization information.

Recently, many studies have shown cell-to-cell variability in gene expression levels in component cells of tissues and solid tumors. For example, solid tumors consist of a collection of many distinct cell types: cancer cells, cancer stem cells, cancer-associated fibroblasts, endothelial cells, pericytes, and immune inflammatory cells, and the microenvironment is also known to influence gene expression in those cells (8). Additionally, cancer cells are not individually same in their differentiated states, proliferation rates, and metastatic potential, which possibly attribute their different sensitivities and responses to drug treatment (7). Therefore, in order to develop diagnostic biomarkers, it is essential to retrieve spatial gene expression information from intact cells and tissues. ISH provides information about heterogeneous expression in single RNA molecules at the single-cell level, which ultimately will be useful for identifying RNA biomarkers with unique profiles in a variety of diseases.

RNA IN SITU HYBRIDIZATION

Since its introduction in 1969 (9), ISH analysis has become an invaluable method for localization and quantification of specific nucleic acid sequences in individual cells and tissues, providing information about tissue-specific, cell-specific, and subcellular gene expression at different developmental stages of biology, and between cells within normal and diseased tissues. ISH is performed, using hybridization procedures between the specifically labeled nucleic acid strand (the probe) and its complementary RNA sequences in fixed tissues or cells, followed by visualization of the target transcript through the radioisotope-labeled or fluorescence-labeled probe, or with immunological detection (10). When ISH was first being developed, radioisotope-labeled nucleotides were used as the gold standard for ISH, with high sensitivity. However, radioisotopes have a long turn-around time, which carries the risk of exposing researchers to radioactivity and radioactive waste disposal (11). ISH underwent further development when it was used in immunological detection. Its hapten-labeled probes and antibody-enzyme conjugates were used in combination with substrates of precipitating chromogens, which has become a widely accepted combination showing comparable sensitivity to radioisotope probes (12). In the late 1970s, fluorescence-labeled probes were then introduced to ISH (FISH: fluorescence in situ hybridization), followed by numerous technical advancements in the engineering of FISH probes and protocols, which improved the resolution, specificity, and speed of FISH (6). Currently, FISH is an invaluable method for the simultaneous detection of multiple RNAs, providing essential information of gene expression in biological science.

SINGLE-MOLECULE FLUORESCENCE IN SITU HYBRIDIZATION

Singer and colleagues developed FISH technology to detect RNA at the single-molecule level, in single cells (13). To generate high-intensity signals from the hybridization of individual RNAs, they used several short probes (50 nucleotides long) that were complementary to sequential parts of the target mRNA and were each coupled to five fluorescent dyes at predefined positions (13). They detected single mRNA molecules as diffraction-limited spots. Thereafter, many different FISH probes have been developed for enhanced detection of single RNA molecules, resulting in robust and sensitive FISH analyses. These single-molecule FISH (smFISH) methodologies have been classified, based on probe designs, as follows: short probes labeled with multiple fluorophores, short probes labeled with single fluorophores, short probes with modified backbones, and signal amplification of single-molecule probes (14). In general, probes that are complementary to different regions of the target RNA molecules are either single or a combination of multiple oligonucleotides, which can be custom-synthesized easily for high specific annealing. smFISH is now a powerful, single-cell transcript profiling method that provides the transcriptional state of any individual cell via quantitation of numerous RNAs in single molecule level (7).

Here, my recent application of smFISH in breast cancer cells is described, and recent improvements and applications in smFISH for functional studies in gene expression are reviewed.

QUANTITATIVE IMAGING OF INDIVIDUAL mRNAs AND CO-IMAGING OF PROTEINS IN BREAST CANCER CELLS

To quantify individual transcripts in breast cancer single cells, smFISH procedures were performed, as described earlier above, using multiple, short probes (20-nucleotide) that were labeled with single fluorophores developed by Raj et al. (15). These probes are commercially available as a ‘Stellaris FISH Probes’ (Biosearch Technologies). Forty-eight different oligonucleotide probes were designed using Stellaris Probe Designer version 1.0 (http://www.singlemoleculefish.com/). Each of these 48 probes had a single fluorophore on its 3’ end, which provided a detectable fluorescent signal when at least 20 probes were hybridized with their complementary sequences in target mRNA, even without an extra amplification step (16). Therefore, potential off-target effects from a few probes, which might cause false positives in the detection of FISH signals, were negligible in this smFISH procedure. Synthesis of short probes is routinely, economically available, and as a consequence, smFISH using short probes labeled with single fluorophores has been applied to a wide range of biological samples (14). Furthermore, the short, 20-nucleotide size allows the probes to access a single target mRNA located in the ribonucleoprotein (RNP) complex. The short probes need much less stringent conditions for hybridization and post-hybridization washing: for the 20-nucleotide DNA probe, 28°C, 30°C or 37°C, and 10% formamide (15-17), instead of 37°C or 47°C, 50% formamide for the 50-nucleotide DNA probe (13, 18), or instead of 65°C and 50% formamide for the classic FISH probe.
of about 500 nucleotides for the RNA probe (19). Standard protocols of IHC are unfavorable both at 37°C or 47°C, 50% formamide, and at 65°C, 50% formamide. However, in less harsh conditions of 30°C or 37°C, and 10% formamide, antibody-antigen interactions can survive since the antibody-antigen interactions are usually mediated by the weaker binding of fewer hydrogen bonds than annealing of oligonucleotide probes to RNAs in FISH. Further, the addition of primary antibodies together with FISH probes can successfully detect proteins and mRNA simultaneously by combining FISH and IHC - a process referred to here as “immunoFISH”. This immunoFISH method has already been used to detect simultaneously exogenously expressed reporter RNAs regulated by miRNA and the process-

Next, smFISH was applied to formalin-fixed, paraffin-embedded (FFPE) breast cancer MCF7C18 cells (Fig. 1C). Three mRNAs (ERBB2, AKT1, and AKT3) were detected in the HCC1954 cell line, using a different fluorophore per mRNA (Fig. 1D). Each RNA particle was also counted, and the number was found to correlate highly with RNA-Seq data.

Interestingly, large groups of ERBB2 RNA signals, referred to as a blobs, were found in the nucleus of HCC1954 (Fig. 1 arrows), which were believed to be aggregates of ERBB2 RNA particles, suggesting a transcription burst of the ERBB2 gene in the ERBB2 transcription site (22). Analyzing RNA molecules in this region was beyond the resolution of this current imaging system. It needed a super-resolution level (10-100 nm range) that would be ideal for analyzing spatial relationships at the ultrastructural level, which so far has remained only in the field of electron microscopy (23). Super-resolution-based smFISH may provide new insights into the functional nuclear organization of gene expression and could help decipher RNA architecture around transcription-burst sites.

To visualize and to quantify Akt signaling and related transcripts in intact breast cancer cells and tissues, immunoFISH...
was performed to simultaneously detect \textit{ERBB2} mRNA particles and phosphoAkt (pAkt), the latter which is an activated form of Akt proteins. \textit{ERBB2} RNA probes and an anti-pAkt antibody, in smFISH conditions of 37°C and 10% formamide were applied. pAkt proteins, detected by fluorescent-labeled secondary antibodies, were found primarily around the plasma membrane (Fig. 2B), a result that was also found when standard IHC was performed. The addition of the primary or the secondary antibody did not affect the counting of mRNA molecules in smFISH and the co-staining of proteins (Fig. 2C).

Using this combined smFISH and IHC, the activated form of mediator proteins (e.g., Akt and ERK) can be simultaneously detected with their downstream endogenous mRNAs, in single molecules at the level of single cells. This method will likely provide a molecular profile of aberrant signaling and the associated transcriptional state of individual cells in breast cancer. It also has a potential application in analyzing biopsies and xenograft tissues of human tumors in order to measure in situ spatiotemporal profiles of aberrant signaling pathways that are activated in cancer.

**APPROACHES FOR SIGNAL ENHANCEMENT**

Increasing the sensitivity of ISH has been a key issue in the quantitative imaging field in order to measure relatively low-copy RNAs and short RNAs, such as miRNAs, especially in FFPE tissues in which the yield of prepared RNA is usually low. To increase the signal-to-noise ratio, several strategies have been developed to intensify ISH signals and suppress background noises. Here recent approaches for signal enhancement are discussed.

**Branched DNA probes**

Branched DNA (bDNA) probes have been used to improve the detection of nucleic acid in ISH, through the application of a series of oligonucleotide probes, hybridized sequentially, in order to generate chromogenic or fluorescent signals (24). bDNA probes consist of four separate probe sets: target RNA-specific probes of 10 or more oligonucleotide, pre-amplifier probes hybridizing to the target RNA-specific probes, multiple amplifier probes hybridizing to the pre-amplifier probes, and labeled probes conjugated to the amplifier probes (14). These probes allow a maximum of 8000 fluorophores to be labeled for target 1-kb RNA (5), which provides massive amplification (167 times more) compared to the maximum of 48 fluorophores associated with multiple, single-labeled probes in the smFISH system. The bDNA probes are commercially available as a ‘ViewRNA’ system (Affymetrix) or a ‘RNAscope’ system (Advanced Cell Diagnostics). However, the huge pre-amplifier and amplifier probes might have difficulty penetrating the complementary sequences of mRNAs that are present in the RNP complex-RNA binding proteins that cause steric hindrance against large probes that target RNA (25).

**Tyramide signal amplification**

Tyramide signal amplification (TSA) is an enzymatic technique widely used in fixed-cell assays, such as immunocytochemistry and in situ hybridization, to enhance sensitivity and specificity (26). TSA-FISH employs DNA or RNA probes that have been labeled with a hapten, such as biotin, fluorescein, or digoxigenin. In brief, the hapten-labeled probe is hybridized to the target RNA, and then fluorescent TSA utilizes the enzymatic catalysis of horseradish peroxidase and fluorescently labeled tyramide substrates, depositing highly dense tyramide species at the site of enzyme activity (27). TSA can improve the sensitivity of FISH over standard methods by 10 to 30 fold (28), even detecting single copy gene (29), which could in turn improve the detection of RNA, even when RNA counts are low, such as in single copy molecules.

**Quantum dots**

Quantum dots (QDs) - nanometer-scale, semiconductor crystallites - have a 10-20 times brighter emission than organic dyes, providing a higher signal-to-noise ratio compared to that in organic dyes (30). Unlike organic dyes, QDs are photo-stable fluorophores due to their inorganic composition that reduces the effects of photobleaching (31). Therefore, QDs are good fluorophores, and they have been used in immunofluorescence to improve signal detection (30), and in a QD-based ISH (QD-ISH) method (18, 32). QD labeling was successfully applied in combined IHC and ISH to detect protein and mRNA at the same time in mouse brain tissue (32).

**Padlock-Rolling Circle Amplification: distinguishing single nucleotides**

Padlock probes were developed in the mid-1990s to detect and to characterize single-copy genes in genomic DNA samples, for example, to analyze the number of gene copies, alleles, and point mutations (33). Padlock probes were also applied to smFISH to detect individual mRNA molecules, in combination with rolling circle amplification (RCA) to distinguish single nucleotides in transcripts (34). Larsson et al. successfully distinguished transcripts that were different only in single nucleotides, such as a single-base difference between human and mouse β-actin sequences (34). In brief, padlock probes are tar-
geted to the cDNA, which is reversely transcribed from the target mRNA in situ; are circularized by high-fidelity ligation; and are amplified by RCA, followed by detection (34). Due to its extreme sensitivity to mismatches at the ligation junction, padlock-RCA can discriminate between wild-type transcripts and point-mutated transcripts in the same cell. To detect individual protein interactions and modifications with single mRNA molecules, padlock-RCA was combined with the in situ proximity ligation assay (PLA) (35). Weiibrecht et al. simultaneously detected phosphorylated PDGFRβ protein and DUSP6/MKP-3 mRNA molecules in individual human fibroblasts upon PDGF-BB stimulation (35). Together with immunoFISH described earlier, padlock-RCA/PLA provides a new methodology to visualize activated signaling pathways and end-point gene activation at the level of single cells.

DETECTION BEYOND THREE SPECIES

The simultaneous detection of multiple molecular species by fluorescence microscopy is limited by the spectral overlap of normal fluorophores and the usual, upper-limit of three different species (36). However, the current study of systems biology requires the simultaneous detection of numerous molecules. The combination of several fluorophores labeled probes per transcript can simultaneously target multiple RNAs beyond the number of fluorophores used (14, 37). In brief, this process involves hybridizing transcripts from different genes with a set of short probes. When a target RNA is hybridized with probes labeled with a single fluorophore, bright singly fluorescent signals are detected, and when a target RNA is hybridized with probes labeled with more than one fluorophore, less bright signals are detected in multiple fluorescent channels (38). In this method, called spectral barcoding, the number (n) of spectrally resolvable fluorophores theoretically provides the number of molecules in RNA according to the calculation $2^n-1$, which encodes transcript identities through a unique combination of fluorophores (14, 39).

Spectral barcoding has been applied with multiple probes per transcript. For the first time, Singer and colleagues analyzed 11 different mRNAs simultaneously at transcription-active sites in serum-stimulated, cultured single cells, resulting in cellular transcriptional profiling with high spatial and temporal resolution (40). Directly barcoding single mRNAs throughout a single cell has not been restricted to a particular transcription site. Jakt et al. (38) used a large number of short, fluorescently labeled probes per target transcript and was able to measure simultaneously the expression levels of more than six genes in cultured mammalian cells, using a combination of FISH with three fluorophores and image analysis. To image single RNA molecules of more than 30 genes at a time, Lubeck and Cai used optical super-resolution microscopy (SRM) and single-molecule FISH to analyze the combined barcode transcripts, taking advantage of the high-labeling specificity of oligonucleotide probes (41). They used spectral barcoding to profile transcripts from 32 stress-responsive genes simultaneously in single Saccharomyces cerevisiae cells (41). They speculated that the implementation of 3D SRM to improve axial resolution, combined with additional fluorophores and computational algorithms, may result in the simultaneous detection of over 1,000 genes (41). This method - of detecting numerous mRNAs by spectrally combining hybridized fluorophore-labeled probes and connecting super-resolution imaging of single cells - promises to provide an economical, powerful tool for transcriptional profiling of single cells in systems biology, without the need to isolate individual cells and to sequence nucleic acids.

QUICK FISH METHODS

FISH has been widely used to analyze gene expressions in ultrastructures of biological systems, while maintaining the subcellular and complex cellular integrity of the systems. However, FISH requires much more time and labor compared to other molecular techniques, such as qPCR and microarray analyses. Recently, new methods have been developed to simplify FISH protocols. The TransISH was reported with one-step signal amplification after hybridization, using new probes without antigen-antibody reactions to detect mRNAs in tissue sections (10). Another FISH method using exciton-controlled, hybridization-sensitive, fluorescent oligodeoxyribonucleotide (ECHO) probes has been developed (42). ECHO-FISH uses multicolor probes in a 25-minute protocol, from fixation to mounting, which does not need stringency washing steps to detect specific DNA and RNA sequences (42). These methods will likely speed up gene-expression analyses and can be anticipated to measure RNAs in single molecules in the near future.

RNA IMAGING IN LIVE CELLS FOR VISUALIZING TRANSCRIPT DYNAMICS

smFISH procedures are performed with fixed cells, which facilitate the quantification and distribution of RNAs in steady state, but they do not provide dynamic, life-cycle information about mRNAs, such as 5’ methyl capping, splicing, polyadenylation, nucleocytoplasmic export, localization, translation, and turnover (43). To analyze RNAs dynamically in the nucleus or cytoplasm of living cells, FISH techniques for detecting endogenous RNAs have been introduced to live cells (44). Politz et al. used oligo(dT), labeled with chemically masked (caged) fluorescein, to penetrate live cells and to hybridize the nuclear poly(A) RNA (45). Laser spot photolysis then uncaged the oligo(dT) at a given intranuclear site. The resultant fluorescent, hybridized oligo(dT) was tracked with high-speed imaging microscopy. This method revealed the movement of endogenous poly(A) RNA in the nuclei of live cells (45). To directly visualize specific mRNAs in live cells using hybridization probes, Tyagi and Kramer developed molecular beacons, which are oligonucleotide hybridization probes.
that generate fluorescence signals only when they hybridize to complementary, nucleic-acid target sequences (46). Molecular beacons resulted in the successful, live imaging of oskar mRNA being transported and localized in Drosophila melanogaster oocytes (47).

Other real-time, nonFISH-based technologies have been developed to measure RNAs in single cells. In 1998, after Bertrand et al. (48) reported their successful attempt to use green fluorescent proteins fused to bacteriophage MS2 RNA coat protein in order to target RNAs, this method has been routinely used to image engineered RNAs containing binding sequences for MS2 coat protein in living cells (49). Application of the Bertrand et al. (48) procedure provides information on the dynamics of single RNA particles in the nucleus or cytoplasm of living cells, and it may provide dynamic images of multiple RNAs, thanks to the development of fluorescent proteins with diverse spectra (50). Recently, Paige et al. generated RNA aptamers that were capable of binding to fluorophores and that mimicked GFP fluorescence (51). They termed the brightest RNA aptamer-fluorophore complex Spinach. Spinach resembles enhanced GFP emitting a green fluorescence that is remarkably resistant to photobleaching (51). Trafficking of Spinach-fused RNAs was successfully imaged in live cells without nonspecific fluorescence or cytotoxicity in cells (51, 52).

CONCLUSION

smFISH provides an extremely sensitive method for visualizing individual transcripts, and for profiling and quantifying multiple transcripts simultaneously in the structural context of single cells. Using smFISH, researchers are able to detect individual RNAs as a sum of fluorescent intensities from multiple oligonucleotides probes that are annealed to complementary RNA in a highly specific manner, without secondary amplification. smFISH takes advantage of molecular biology technologies and nanotechnologies through the use of probes comprised of bDNA, padlock-RCA, and QDs, resulting in the detection of single transcripts in histopathological samples. Thanks to advancements in imaging systems, numerous RNAs can now be measured by combining super-resolution microscopy and spectral-barcode labeling (41). smFISH has already been combined with IHC to monitor the activation of signaling proteins simultaneously with regulated transcripts. ImmunoFISH could be combined with multicolor DNA FISH to visualize interphase chromosomes in their entirety as well as in their transcription states that have been associated with the movement of loci into and out of nuclear subcompartments (53). These combined technologies promise to provide researchers with a molecular profile of healthy and of aberrant cells, eventually resulting in a better understanding of how chromosomal localization of DNA affects transcriptional activity and how subcellular localization of RNA affects translational activity.

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