CHAPTER TWENTY-SEVEN

# Cell Biology of mRNA Decay

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**Abstract**

Studying single mRNA molecules has added new dimensions to our understanding of gene expression and the life cycle of mRNA in cells. Advances in microscopes and detection technology have opened access to single molecule research to most researchers interested in molecular biology. Here we provide an overview technique for single molecule studies of RNA in either fixed samples or in living cells. As part of a volume on mRNA turnover, it is increasingly relevant, because many of the recent advances in studies of mRNA turnover have suggested that there is non-homogeneous distribution of turnover factors in the cell. For this reason, understanding of spatial relationships between mRNA and mRNA turnover factors should enrich our understanding of this process.

## 1. Introduction

Studies of mRNA localization within the cytoplasm have driven the development of microscopy techniques to evaluate the spatial distribution of mRNA within the cell. Consequently, these discoveries have also made the techniques more relevant to studies of mRNA turnover. The discoveries of processing (P)-bodies in yeast and similar P-bodies or GW bodies in mammalian cells, as well as stress granules in mammalian cells, have increased the importance of collecting spatial information for understanding the processing and degradation of mRNA within the cytoplasm of eukaryotic cells (Anderson and Kedersha, 2008; Garneau *et al.*, 2007; Kedersha and Anderson, 2002; Parker and Sheth, 2007; Sheth and Parker, 2003). P-bodies and stress granules are observed in the eukaryotic cytoplasm as local enrichments of factors that are involved in the processing of RNA, many of these being factors involved in mRNA degradation (Eulalio *et al.*, 2007; Fenger-Gron *et al.*, 2005; see chapters by Nissan and Parker; Kedersha and Anderson). Similar observations have been made in nuclear processing and turnover pathways (Houseley *et al.*, 2006; LaCava *et al.*, 2005; Wagner *et al.*, 2007). In each case, mRNA is likely to interact with the proteins found within these structures, but how the mRNA interaction with these structures contributes to regulation of mRNA in the cytoplasm is poorly understood. Therefore, studies of individual mRNAs should contribute significantly to studies of these structures and other processes regulating mRNA.

When an entire culture of cells or a piece of tissue is homogenized and extracted to isolate RNA, DNA or protein, subpopulations are averaged and only observations occurring within a significant proportion of these

cells in the whole population can be detected. The most widespread approaches for studying individual macromolecules rely on examination of the pooled samples of these molecules from whole cultures or tissue. These approaches have limitations when only subpopulations are being sought, such as cells in some particular phase of the cell cycle, or some specific cell type within a tissue (Levsky and Singer, 2003). Sorting individual cells within these cultures enhances the ability to look at subpopulations of cells; however, the requirements for large numbers of cells limit this approach when cells of interest are sparse. Cultures can be manipulated to enrich for subpopulations, but these manipulations may have cryptic effects on the results obtained. For these reasons, microscopic analysis of individual cells has become commonplace to examine cellular phenomena. The ability to examine particular subpopulations of cells in a population with markers allows one even to evaluate cells that are rare within a population.

These same advantages of microscopic analysis use can be applied at the molecular level with single molecule imaging approaches to evaluate populations of molecules individually rather than in bulk. One major advantage to this approach is that spatial information is retained that would normally be lost on sample homogenization. As more sensitive quantitative microscopy equipment becomes accessible to most researchers, it is feasible for most laboratories to perform single molecule analysis, and RNA is one of the most amenable subjects to apply single molecule approaches. With *in situ* hybridization using fluorescently labeled oligonucleotide probes (fluorescence *in situ* hybridization, FISH), one single protocol is flexible enough to examine almost any RNA species microscopically. Any unique RNA sequence can be examined by synthesis of a complementary deoxy oligonucleotide probe. The availability of many spectrally distinguishable fluorophores even allows examination of many different mRNAs within the same cell (Capodieci *et al.*, 2005; Levsky *et al.*, 2002).

Our laboratory has previously established methods for detecting RNA molecules in fixed mammalian cells and tissues with FISH and has shown that the technique can successfully detect individual mRNAs with commonly available microscopy equipment and software (Capodieci *et al.*, 2005; Femino *et al.*, 1998; 2003; Shav-Tal *et al.*, 2004). We refer readers interested in detailed description of FISH to these articles to become more familiar with the approach. In the first part of this article, we will provide a brief presentation of the important aspects of single molecule mRNA detection with FISH for mammalian cells and present a basic protocol. More details about the protocols for FISH can be found in the previous references and at the following url: www.Singerlab.org. Recent years have seen the development of techniques for visualizing single mRNAs in real time in living cells, and we will devote the second half of this chapter to the analysis of live cell single mRNA detection.

## 2. FISH Probe Design

The first step is to design probes for hybridization. Oligo DNA probes are our preferred probe; therefore, the protocol presented here is designed for such probes. Parameters for probe design have been previously elaborated, and our standard FISH protocol has been worked out with 40 to 50 mer oligonucleotide probes that have a 50% G–C base pair content. Higher or lower GC content can be accommodated if need be but will necessitate changes in hybridization temperature or formamide concentration that should be empirically determined. When hybridizing multiple probes, it is simplest to have all probes with very similar GC content so that all the probes can be hybridized in one reaction. Oligo probe sequences should be selected such that there is no extensive complementarity to other RNA sequences in the transcriptome, and advances in genomic databases have made the selection of very specific probes more attainable. Sequences against exons will hybridize to mRNA everywhere; however, sequences against introns can be used to very specifically localize sites of transcription, because this sequence should only be present in the nascent transcript before splicing (Zhang *et al.*, 1994).

The probes are detectable by the covalent modification of the synthesized DNA oligo with fluorescent dyes. We have used two methods for labeling probes. The first is to synthesize the oligo probe with modified amino-allyl T residues spaced approximately 10 to 15 nt apart. This spacing is important to prevent quenching of fluorophores that are too close but to accommodate enough dyes to detect a single probe clearly. In this way, a sequence can be modified with three to five fluorophores. Labeling with three dyes has been shown to be sufficient, depending on the dye, but results from a single probe are more robust with five dye labels. A single modified T residue that is not complementary can be added at either the 5′ or 3′-end without affecting the hybridization of the whole probe, increasing flexibility of choosing the probe sequence. After synthesis and probe purification, the amino-allyl T residues are available to any dye that can couple through the free amine group, and most commercially popular dyes are available ready for amino coupling. Many scientific reagent suppliers now offer oligos prelabeled with fluorophores; therefore, oligo probes can be synthesized with the fluorophore, eliminating a separate labeling step. Although there are limitations to the numbers of fluorophores that commercial providers can add. When designing probes to be used against multiple, different RNA targets simultaneously, ensure that the two different probes are each labeled with fluorophores that have nonoverlapping spectra. Efficient probe labeling is a critical parameter for robust FISH detection. Significant populations of poorly labeled or unlabeled probes will compete directly with well-labeled probes,

reducing the amount of mRNAs that can be detected by the technique. For negative controls, a no probe FISH can control for autofluorescence, and a 50-mer randomized probe sequence with evenly spaced amino-ally T residues can control for nonspecific probe binding.

## 3. Hybridization

The procedure for FISH is quite similar to that for standard immunofluorescence (IF) and, therefore, should not require any specialized equipment that cannot be found in any typical molecular/cellular biology laboratory. However, specific nucleic acid hybridization necessitates quite different buffer conditions than IF. For the 50% GC content probes, we present a basic protocol that can be used for the *in situ* hybridization. This protocol can be optimized for alterations in GC content by changes in formamide concentration or temperature. Higher GC contents may require either higher formamide concentrations or temperature of hybridization, and, conversely, lower GC contents may require lower formamide or temperature of hybridization. In cases in which GC content for multiple probes to be hybridized to the same sample cannot be similar, it should be possible to perform two sequential hybridizations, with the higher stringency conditions being performed first, followed by the lower stringency conditions.

For studies of mRNA localization, the distribution of individual mRNAs can be described from these images, and the influence of experimental variables on this distribution can be analyzed in parallel samples. Similarly, by use of IF-FISH as described in the following, the spatial distribution of mRNA can be analyzed quantitatively in relationship to cellular landmarks that are colocalized with the IF channel. The spatial relationship of mRNAs relative to protein markers for particular cellular structures can also be measured under different experimental conditions to examine the effect of these variables on interaction of mRNA with these structures.

## 4. Image Acquisition

The signal of three to five fluorophores on a single oligo DNA probe should be sensitive enough to clearly observe FISH signal, but the intensity of these signals may, nonetheless, not be very high above the autofluorescent background of the cell. This may result in a skewed appearance of the raw FISH image, because photons that come from autofluorescence and from out of focus probes can add to the photons in voxels containing the

FISH probe. In this way, regions of the cell with higher autofluorescence (which is not typically uniform throughout the cell) or concentrations of FISH probe may appear to have more intense mRNA particles than regions with lower autofluorescence or more sparse distribution of mRNA. However, the photons derived from the FISH probes in these regions are equivalent, but may not appear so, making it a challenge to find an appropriate scale to display images. For this reason, deconvolution is used to analyze FISH images, because the difference between the autofluorescence and FISH probe point signals become more apparent. Deconvolution is a mathematical algorithm designed to correct for the unavoidable distortion of the optics (Wang, 2007). The algorithm uses the point-spread function of the optical system to remove out-of-focus light, which reduces noise. Constrained iterative deconvolution algorithms will reassign the out-of-focus light to its source position, thereby improving signal. Several commercial deconvolution software packages are available. One should acquire serial optical sections throughout the Z-axis of the cell to perform deconvolution.

To acquire images for quantitative analysis, exposure times and excitation intensities for each channel must be carefully set independently, such that the images for the FISH and the negative controls are comparable. The exposure time and intensity required to obtain significant signal from the hybridized samples can be determined first, and the no-probe containing samples can be acquired under the same excitation and exposure conditions. Both control and FISH samples can be deconvolved, and the resulting images analyzed; 60× and 100× magnification high numerical aperture (>1.3 N.A.) objectives combined with standard scientific grade cameras that have pixel size between 6 and 8 $\mu$m provide sufficient spatial resolution for image analysis, 100× providing slightly higher spatial over sampling.

## 5. FISH Protocol

This protocol has been established for cultured cells grown on glass coverslips. Fixation with 4% paraformaldehyde (PFA) is compatible with FISH, but most routine methods of fixation are also suitable. We routinely use alcohol permeabilization after fixation (80% methanol or ethanol), because samples can be stored for days at −20 °C after this treatment, but detergent permeabilization works too if cells will be used immediately. Extended storage of fixed cells may result in loss of hybridization sites. The FISH step will prevent phalloidin staining, so this reagent cannot be used to label actin filaments. DAPI remains effective after FISH. When performing FISH on samples expressing fluorescent proteins, both alcohol permeabilization and the FISH buffer will denature fluorescent proteins, eliminating the fluorescence produced by the protein, but the fluorescent

protein can still be detectable by IF with primary antibodies against the fluorescent protein that are compatible with FISH. (See IF-FISH protocol.)

## 5.1. Probe mixture

50 μl of probe mixture is enough for one 18-mm coverslip
24 μl formamide
24 μl 20% dextran sulfate in 4× SSC
0.5 μl 20 mg/ml BSA fraction V (or acetylated BSA)
0.5 μl 10 mg/ml sheared salmon sperm DNA
0.5 μl 10 mg/ml *Escherichia coli* RNase-free tRNA
0.5 μl 10 ng/μl labeled oligonucleotide probe (for multiple probes one mixture of all probes at 10 ng/μl for each probe)

## 5.2. Preparation of cells

Fix: 20 min in 4% PFA/PBS
Quench: 20 min in PBS w/0.1 *M* glycine (PBSG)
Permeabilize: 80% methanol 10 min
Rehydrate: 6 serial twofold dilutions of the methanol in PBSG
Final rinse 5 min in PBSG
Equilibrate in 50% formamide/2× SSC (2× 5-min incubations)

## 5.3. Hybridization

Denature probe by heating at 65 °C for 5 min.
Hybridize: Place coverslips face down onto the probe solution spotted onto a strip of parafilm in a *humidified chamber* to prevent evaporation.
Incubate 37 °C for 2 h.

## 5.4. Wash

Transfer coverslips face up into the wash vessel with 2× SSC/50% formamide at 37 °C.
10 min at 37 °C in 2× SSC/50% formamide.
Change buffer and incubate another 10 min at 37 °C.
Change buffer to 1× SSC/50% formamide prewarmed to 37 °C.
Wash 15 min 37 °.
Change buffer and wash an additional 15 min.
Change buffer to 1× SSC (no formamide, room temperature).
15 min room temp.
Change buffer to 0.5× SSC.
15 min room temperature.
Add 0.5× SSC with and DAPI.

5 min room temperature.
Rinse 3×, 1 min each with 2× SSC.
Mount coverslips in mounting medium and follow the appropriate instructions for the mounting medium used, then the cells are ready for imaging.

### 5.5. Preparation of humidified chamber

To prepare a simple humidified chamber, spread a piece of parafilm in the bottom of a plastic culture dish.
Place the probe solution in one drop per coverslip on the parafilm; leave enough distance between so that coverslips will not contact each other during incubation.
Place coverslips face down onto drops of probe.
Add a falcon tube capful of PBS in the corner to keep humidity in the chamber during incubation.
Place the lid on the culture dish and seal the vessel by wrapping with parafilm around all the edges.

## 6. Colabeling Protein with IF and RNA with FISH

FISH is also compatible with colabeling by immunofluorescence; however, the potential for RNase contamination in antiserum or antibody preparations necessitates very careful control of the FISH steps, because degradation of the RNA during any step will result in the loss of hybridization sites in the fixed cells. Ribonucleoside vanadyl complex (RVC; New England Biolabs Inc.) is an effective inhibitor of RNase activity residing in serum or antibody preparations, but these must be used according to the manufacturer's instructions, paying attention to the effective concentrations, methods for resuspending them, and buffer conditions (these are inactivated by EDTA). If RVCs cause some low-level background, adding EDTA to the final washes may eliminate this. When performing IF and FISH simultaneously, the high concentration of formamide is denaturing to some antigens. The order of steps is flexible but will be dictated by the antigen–antibody combination. The potential to lose antibody or target antigen during the harsh FISH step may not be compatible for some antibody–antigen combinations. In this case, a brief PFA cross-linking step after primary antibody incubation can help retain IF signal during FISH. However, for each particular antigen-antibody that works in IF, we suggest that the IF steps without the FISH hybridization steps be run on a parallel sample to control for the loss of antigen during the FISH step when performing IF-FISH. We present a basic protocol for IF-FISH that performs the primary antibody incubation, followed by a brief cross-link

and then FISH, followed by the secondary antibody before mounting and imaging cells. The protocol allows for the primary antibody incubation to occur as normal before the use of the harsh FISH conditions to minimize damage to sensitive antigens and facilitate their detection. Good antigen–primary antibody combinations can be probed after the FISH is performed first; however, contaminating RNase activity can release hybridized signals and must still be controlled. The secondary antibody incubation is performed subsequent to FISH to prevent denaturation of the secondary antibody during FISH, including RNase inhibitors in case secondary antibody is also contaminated with RNase activity.

## 7. IF-FISH Protocol

This protocol uses fixed cells prepared as earlier for FISH and the same probe solution. RNase inhibitor (RVC) is added to all IF incubation solutions to control for potential RNase contaminations that will eliminate the FISH signal. CAS block (Zymed/Invitrogen) is a commercially available blocking agent that is compatible with FISH. Because this is not routinely assayed for RNase activity, we add RVCs to this during blocking, and any typical blocking solution should work with RVCs added.

### 7.1. Primary antibody incubation

(This protocol starts after rehydration of the methanol permeabilized cells earlier.)

Block: CAS block with 10 m*M* RVC for 30 min at room temperature.
Primary: Incubate coverslips with an appropriate dilution of primary antibody in CAS block with 10 m*M* RVC (timing and temperature conditions appropriate for particular antibody-antigen combination).
Wash: 10 min PBST (PBS with 0.1% Tween 20).
Fix primary: 10 min PBS-4% PFA.
Quench: 20 min PBSGT.
Equilibrate in 50% formamide/2× SSC (2× 5-min incubations).

### 7.2. FISH hybridization

(Probe preparation and FISH is as described in section 5, FISH protocol.)

Denature probe by heating at 65 °C for 5 min.
Hybridize: Place coverslips face down onto the probe solution spotted onto a strip of parafilm in a humidified chamber to prevent evaporation.
Incubate at 37 °C for 2 h.

### 7.3. FISH wash

Transfer coverslips face up into the wash vessel with 2× SSC/50% formamide at 37 °C.
10 min at 37 °C in 2× SSC/50% formamide.
Change buffer and incubate another 10 min at 37 °C.
Change buffer to 1× SSC/50% formamide prewarmed to 37 °C.
Wash 15 min 37 °C.
Change buffer and wash 15 min more.
Change buffer to 1× SSC (no formamide, room temperature).
15 min at room temperature.
Change buffer to 0.5× SSC.
15 min room temperature.

### 7.4. Secondary antibody

Secondary: Incubate coverslips with an appropriate dilution of secondary antibody in CAS block with 10 m*M* RVC (timing and temperature conditions appropriate for particular secondary antibody).
Wash: 4× 15-min each with PBST (add 1 m*M* EDTA if RVC background is observed).
DAPI: 5 min in PBST with DAPI.
Wash 3× 1-min with PBS.
Mount coverslips in mounting medium and follow the appropriate instructions for the mounting medium being used, then the cells are ready for imaging.

## 8. Following mRNA in Living Cells

The use of fluorescent proteins has allowed researchers to observe the behavior of proteins in real time in living cells. Exploiting very specific RNA–protein interactions has enabled application of fluorescent protein technology to studies of RNA by introducing tandem RNA tag sequences that can be specifically recognized by corresponding fluorescent protein chimeras (Fusco *et al.*, 2003; Janicki *et al.*, 2004; Shav-Tal *et al.*, 2004; Bertrand *et al.*, 2008). The concentration of these chimeras on coexpressed tagged RNAs enables microscopic visualization of the mRNA in real time in the living cells. Similar to the FISH methods, we have recently published detailed descriptions of the use of the bacteriophage MS2 coat protein (MCP) as a fluorescent probe for tagging mRNAs in living cells and suitable microscopic requirements for these experiments (Chao *et al.*, 2008; Wells *et al.*, 2007). The MCP fused to a fluorescent protein (MCP-FP) can bind an

RNA sequence from the MS2 genome (the MCP-binding site, MBS). When the MBS is multimerized, many copies of the MCP-FP are targeted to an individual mRNA, and the label is significantly stronger than the background of individual MCP-FP monomers. Because we recently published detailed descriptions of this system, we suggest readers refer to those articles for detailed descriptions and protocols.

The primary data that are acquired from these types of experiments are time-lapse images (movies) of the mRNA signal in the cell. Critical in being able to generate movies of mRNA behavior is to obtain sufficiently strong signal from the multiply tagged mRNA target to detect the mRNAs as distinct particles above the background of untethered fluorescent protein probes consistently over multiple frames. The next critical parameter is the acquisition time. mRNA particles are likely to be moving within the cell, either by diffusion or directed, and to successfully generate movies that can capture these movements, exposure times need to be short and frame rates fast enough to capture these particles over multiple frames. Longer exposures and slower frame rates have the effect of allowing the faster moving particle to blur over many pixels, thus only slower moving particles can be reliably analyzed, and the fastest moving population of particles is missed. The ultimate goal of these efforts is to achieve movies from which temporal measurements can be obtained. We have only summarized the protocols for FISH in the first part, because there are several detailed articles regarding this technique in press. However, because the technical parameters to use this approach for live imaging of mRNA are not as well described, we provide a more detailed discussion of this than in the first part. We will discuss parameters that can be extracted from these movies that apply to the MCP-MBS system, but these approaches apply to analysis of any moving particles.

## 9. Live Single-Molecule Detection

The observation of single molecules has become a beneficial tool for biologists during the last decade, and it has been demonstrated that single particle tracking can resolve diffusion times of proteins similar to fluorescence correlation spectroscopy (Grünwald *et al.*, 2006a; Zlatanova and van Holde, 2006). Many assorted techniques are used in single molecule studies, and some are more suited for work in living cells than others (Serdyuk *et al.*, 2007). Of special interest in cell biology are fluorescence-based approaches because of easy application to fixed and live cells in culture allowing observation in the native biologic environment of the molecules under study (Grünwald *et al.*, 2006b). In both scenarios (fixed and live cells), observation of single molecules allows one to extract quantitative data about

the number of observed molecules and their position in three-dimensional space. Counting the number of individual molecules is done by summing up the total of all observed signals, and intensity analysis allows one to determine how many molecules are present within each observed signal. Signal intensity is directly proportional to the number of single molecules present within a discrete signal only when the probe signal intensity is homogenous and interaction between probe and target is uniform and specific. The positions of each discrete signal within the 3D space can be extracted by fitting the intensity distribution, and methods for this have been described in the literature (Schmidt *et al.*, 1996; Thompson *et al.*, 2002). As long as discrete signals are sufficiently separated in space (i.e., no overlap of signals within the limits of optical resolution), these can be reordered in a spatially separated manner, and the fitting process will report their position with sub-wavelength accuracy. For a detailed discussion see Thompson *et al.* (2002) and Yildiz and Selvin (2005). The use of multiple colors (multiplexing) in single molecule experiments promises new quantitative accuracy for colocalization data.

The analysis of live cells makes it possible to study the biogenesis and decay of single molecules. Imaging fixed cells provides a clear precision in determining localization within 3D space at an instant in time, because subjects are not moving, but imaging of single molecules within living cells offers the added benefit of observing behavior (transport, diffusion, confined mobility) of the molecules. Also, single molecule analysis in real time circumvents the problems inherent with analyzing ordered processes in heterogeneous cell populations, where a typically ordered series of events under study are initiating and proceeding asynchronously throughout the population. These individual events can be visualized under more physiologically relevant conditions than if artificial conditions need to be enforced to synchronize all of these events within the population. Because each observation is made on its own time scale, it is possible to pool data from many single molecule events adding significant statistical relevance.

## 10. Single mRNA Data Analysis; What You Can Observe

The ultimate goal of most single molecule live experiments involves the tracking of an individual molecule for a given time to describe its behavior. For instance, the dynamic behavior of an RNA molecule can be tracked as it diffuses away from the transcription site in the nucleus, exports through the nuclear pore complex (NPC) into the cytoplasm, transports to discrete cytoplasmic locations, and finally degrades. Technologies for single molecule imaging in live cells are still developing, and even

more challenging is extending these technologies to studies of RNA. The major limitation in single molecule imaging is the detection of signal. The available signal from a single molecule depends on the intensity of the fluorophore it carries. Efficiency of target labeling, photobleaching during fluorescent excitation, and, in live cell experiments, proper exposure time to allow imaging of the moving particles, all present limitations to establishing single molecule sensitivity. Adding multiple labels can decrease the detection time but must be weighed against the possible influence of the label on the observed behavior and function of the labeled molecule. This is in contrast to fixed cells where no dynamic processes are observed and the exposure time is limited by photobleaching. For dynamic processes, such as can be observed in living cells, the integration time is necessarily dictated by the kinetics of the process under observation. For slow-moving processes, longer integration times are possible, but for a process (e.g., free diffusion) that involves rather fast motion, very short integration times (<100 msec) may be necessary.

Several parameters can be obtained from even simple time-lapse observations of single molecules. In analyzing movements from single focal planes, extended single path traces are often difficult to obtain in many cell types because of movement of particles out of the focal plane, but jump distance (the distance a particle travels between consecutive frames) is simple to derive, requiring only a few frames of continuous observation, and collecting multiple events delivers the necessary statistics. Dwell time is the amount of time a molecule spends in one place, for example, the amount of time a molecule remains associated with the NPC while it traverses the nuclear envelope during export (Kubitscheck *et al.*, 2005). Analyzing multiple individually observed molecules allows one to build a histogram of the timing to describe the average timing of the event. The particle brightness as measured by fluorescent intensity is another parameter that can easily be quantified for multiple objects and plotted to collect many individual particles. Several criteria that we will discuss next are required to extract valid data from these experiments.

For single traces, a detailed analysis showed that the variance of the mean value is a function of the square root of the number of measured positions (Qian *et al.*, 1991). Therefore, traces must be observed through enough frames to become statistically significant for mobility patterns to be evaluated. To obtain an accuracy of 10%, Qian *et al.* suggest a ''trace length of 100 observations.'' This limit can be reached for processes that are bound to a two-dimensional structure or in very flattened out cellular cytoplasm. But because rapid observation in cells is often limited to a single focal plane, it is much harder to fulfill this requirement, because molecules can move in all three dimensions, including out of the focal plane. The signal of a particle fades into noise very rapidly when it moves out of the focal plane. However, very bright particles can cast light into multiple focal planes. Hence, in

single molecule imaging, the effective thickness of the focal plane depends not only on the objective but also on the signal intensity of the single molecule (Kues and Kubitscheck, 2002).

Imaging a single molecule for a sufficiently long time is not only necessary to evaluate its mobility, but even more important for determining many biological functions. For many cell biological questions, the ultimate application of single molecule tracking with live cell microscopy is to image a complex, multistep process, such as the lifetime of an RNA from its birth at the transcription site in the nucleus to its degradation in the cytoplasm. One major obstacle in imaging such a process includes photobleaching the fluorescent label before the molecule reaches the final stage of its journey. Another challenge is to optimize the probability of tracking the molecule in 3D space. In fact, the higher the mobility of the molecule under observation, the faster it will move out of the focal plane (Kues and Kubitscheck, 2002). A molecule with a mobility of 1 $\mu m^2$/sec has an average jump distance of 550 nm in any direction within 50 msec. Detecting enough signal within a single visual plane to visualize this molecule over this time is already difficult, but taking serial optical z-sections on the same time scale to capture the z movement of the molecule can become limiting for the experiment. When interactions between a single molecule of interest and a structure (e.g., P-bodies speckles or the NPC) are observed, the less mobile entity will limit detectable movement. This can help determine the dwell time of the more mobile elements by restricting observation to the volume containing the less mobile structure of interest.

## 11. How Do You Know That You See Single Molecules?

Commonly, three criteria can be used to support the observation of single molecules. These are digital *bleaching*, *blinking*, and *intensity* calibrations. If a signal of a particular intensity disappears in one step (digital, meaning either on or off), the source of the signal must have been a single emitter. Digital bleaching of single fluorophores occurs in one step. Blinking has been used to argue for the observation of single quantum dots, where fluorescent emissions cease temporarily before being observed again. Working with single emitters in fixed cells, blinking and/or bleaching will provide valuable evidence supporting the observation of single molecules. Enhancing the signal by multiple labeling is a powerful strategy to improve signal intensities for imaging. Here, bleaching can still provide good support if multiple irreversible stepwise decreases of fluorescence intensity are observed. Observing a subsequent stepwise bleaching of immobilized fluorophores known quantities of multiple labels is a strong indicator for

observing single molecules. When multiply labeling, autoquenching of dyes is possible, and, if so, the total steps observed for a given molecule might not represent the total labeling ratio.

These criteria are challenging to meet during live observation, because a particle that leaves the focal plane will leave the same signature as a bleached emitter. A moving emitter that blinks will not be seen while in the dark state and cannot be analyzed. To calibrate mobile particles, intensity calibrations can be used to identify single molecules. For calibration of fixed samples, such as described in the FISH section, individual probes can be immobilized to measure the average intensity, and then the average intensity of this signal is used to generate a fluorescence intensity standard curve against which to compare mobile molecules. A slightly lower intensity for these molecules during live imaging is expected, because their movement will blur their signals during image acquisition. With one or few fluorophores, this intensity calibration approach works well as in FISH quantification. However, the use of many fluorophores introduces much more variation to the intensity averages of single particles and, therefore, presents challenges for calibrating the genetically encoded MCP-MBS particles. In this case, an intensity distribution–based argument can be made. Digital photobleaching is accepted as support for observing single dyes. If the intensity distribution of all the observed objects in a field shows discrete steps separated by uniform integral intensity differences, this argues for the presence of a fundamental base unit, and the steps then most likely represent multiples of this base unit.

Can this be used to argue for the observation of single molecules with a uniform multiplexed label? If sensitivity does not allow detection of single molecules, the observed base intensity (the lowest intensity value of all observed objects) could be any multiple of the true single molecule. However, the least complex possibility is that the lowest intensity particles are, in fact, single molecules when sensitivity is sufficient to detect them. If this is true, objects observed in the same frame or movie with an intensity that is an integral multiple of the lowest intensity found must be complexes of the exact stoichiometry that agrees with this integral difference. In plain English if the lowest intensity objects are singles, then objects twofold this intensity are dimers, threefold this intensity are trimers, etc. With multimeric single molecules (e.g., proteins that exist constitutively as multimeric) then this property will obviously be inherently present in the value of the base unit. Nonetheless, all such objects will still be equivalent in intensity. When the nature of the molecule under study is unknown, then this possibility merely leads to some uncertainty about the nature of the single molecule, not that it is a single molecule. mRNA does not multimerize nonspecifically, although complementary sequence can lead to dimerization in some cases. FISH studies have confirmed that an individual mRNA species does not generally

multimerize (Femino *et al.*, 1998). Therefore, there is precedent that the base unit for MCP-MBS labeled mRNA is, in fact, single species.

A potential weakness of the argument with integral multiples comes from the imaging method. Brightly labeled single molecules by multiplexing its labels increases the focal depth that the particle is observable in a far-field microscope. Bright complexes that are out of the focal plane could have the same or any intermediate intensity. As a consequence, z-sections and photon reassignment could be used to minimize artifacts as in deconvolution for FISH (Femino *et al.*, 1998). Alternately, 3D tracking (Levin and Gratton, 2007) or optical sectioning would help. The use of confocal or TIRF microscopes could provide such sectioning (different microscopes for single molecule detection will be described later). By use of an epi-illuminated microscope, blurring of out-of-focus signal may be used to define a threshold for a diffraction-limited signal that has a higher probability of originating from the optical plane of the objective. Theoretically, it is even possible to analyze diffraction patterns to determine the exact 3D position (Speidel *et al.*, 2003).

The imaging, and especially the dynamic imaging of single molecules, requires a critical reevaluation of the way most biological imaging is performed. How many molecules can be tracked at the same time? How fast do they exchange? How precisely can their positions be determined? How much signal must be integrated? How long can a single molecule be imaged before it bleaches? What time resolution is needed to track them? What signal sampling is required? There are no uniform answers to these questions, because each experiment will require optimization of these parameters. To keep the tone of this chapter consistent, we summarize a descriptive framework as an appendix to this chapter that should help researchers in finding reasonable start parameters on the basis of established principles. The appendix also contains a separate section on suitable controls for microscope function and data acquisition, which are important, because microscope stability can have a large impact on the results of single molecule experiments.

## 12. The Secret to Getting Good Data: More Photons, Less Noise

The best images for extracting the types of data discussed require low background and as high a signal as possible at sufficient sampling rates in both time and space. These experiments face limited observation area and low signal intensities as major challenges. Reducing background signal and increasing photon output of the fluorescent labels may improve the signal available from single molecules. Under these conditions it is also important to recognize that too many molecules within a volume field hinder efforts to

analyze individual molecule behavior. With the extremely sensitive detection required for detecting single molecules, signal produced from out-of-focus particles can significantly add to the background of any image; therefore, it is important that the proper number of molecules is present to be able to image them. Use of laser light is very beneficial, as will be discussed in the technical section, but adds to the cost of the setup.

## 13. Setting Up a Microscope for Single Molecule Detection

Point scanning confocal, spinning disc confocal, TIRF, and epi-illuminated microscopes are the most common equipment available in imaging facilities or laboratories. Depending on the experimental conditions, each of them has specific advantages. Detailed discussions of these advantages can be found in the literature (Pawley and Masters, 2008). Confocal setups provide contrast enhancement and hence SNR improvements, because they reduce out-of-focus background in the image with a pinhole or slit. Although point scanning leads to either long integration times or very short excitation of each pixel, spinning disc or slit-based confocal microscopes are faster while offering longer integration times per pixel. New resonant scanners can be used for very fast point scanning but do not change the dwell time of the laser on each pixel. Spinning disc confocal microscopes, TIRF microscopes, and epi-illuminated microscopes use CCD cameras for detection. The quantum efficiency of these detectors can be significantly higher than 90% and hence better than the efficiency of photomultiplier detectors used in point scanning microscopes. Although spinning disc imaging allows optical sectioning, TIRF and epi-illuminated microscopes will be more sensitive for signal detection. Descanning optics (these are the optical elements necessary to guide the detected light to the detector with the scanning element) in confocal and spinning disc microscopes limit the total transmission efficiency, whereas TIRF and epi-illuminated microscopes can be setup with a minimal number of lenses consisting of the objective and a matched tube lens. Although TIRF microscopes provide outstanding signals close to the cover glass surface, the penetration depth of the evanescent field is limited to approximately 100 to 200 nm above the glass surface. Low angle oblique or highly inclined thin illumination (Sako and Yanagida, 2003; Tokunaga *et al.*, 2008) can be used to increase the z-axis penetration depth of the excitation light but even epi-illuminated alignment is possible.

Standard epi-illuminated microscopes often already exist in a laboratory and can be upgraded for single molecule work. The technological step from normal fluorescence imaging to imaging of single molecules can be rather

small and will depend mainly on the brightness of the single molecule signal and the required acquisition speed. Most laboratory front-illuminated CCD cameras with low noise over long integration times can be used for imaging of fixed cells and multiplexed labeling of single molecules. The use of sputtered fluorescent filter sets can enhance the signal-to-noise ratio, because these filter sets have nearly perfect transmission characteristics. The next step of improving such a setup is to add a highly sensitive back illuminated CCD. Recently, electron multiplying (EM) has been introduced as a standard feature for fast and sensitive CCDs by all major companies. These EMCCDs provide a major step in detection sensitivity, but because most are based on a 16-$\mu$m pixel size, a standard 512-pixel chip is already approximately 8 mm in size. For precisely localizing single molecules with 60× or 100× objectives, these CCDs do not provide Nyquist sampling. This can be addressed by use of a magnification lens in front of the CCD, by adjusting the focal length of the tube lens, or by use of additional magnification provided by the microscope stand (e.g., the 1.6× magnifying lens on the microscope stand).

If these changes do not result in strong enough signals (e.g., because the required acquisition time is short), changing the excitation source can be beneficial. Although fluorescence lamps deliver a total of 50 to 300 W over the entire spectrum, their effective power at the sample is limited by the excitation filter that allows only the needed spectral region. Although the total power is given for the whole spectrum of the bulb (approximately 250 nm to 1100 nm), the power resulting from any 40-nm wide band-pass filter will be only a couple of mW. Although many single emitter experiments are done with excitation powers in the kW/cm$^2$ range, the power of a lamp-based fluorescent microscope will be in the W/cm$^2$ range. For very sensitive imaging, background reduction is often more efficient than boosting the signal. Any excitation filter will have a certain not negligible band-width introducing a low additional background. In this sense, even the background of the excitation filter (which is due to very good but imperfect optical densities of the filter for the blocking range) can be a problem. The use of laser excitation is advantageous because the band-width of the excitation light is in the sub-nm range, eliminating stray light from non-exciting wavelengths. Moreover the power delivered to the microscope is almost identical to the output power of the laser, and by the use of high-magnification objectives, the applied power at the sample can be adjusted to the kW/cm$^2$ range while excitation background is reduced to a bare minimum. Laser merge modules are commercially available but costly. For integration of a laser into an imaging system, it is necessary to have fast enough shuttering ability. The use of acoustic-optical devices such as acoustic-optical tunable filters (AOTF) allows for precise control of the excitation light. It is important to understand that, whereas a fluorescent lamp will be collimated to provide a nearly homogenous intensity distribution for the field of view, a laser will come with a narrow Gaussian beam

shape. Flat top optics or expanding the beam with an adjustable iris to limit the illuminated area can be used to provide a homogenous excitation profile. Although the flat top optics are costly, they provide the benefit that the total loss of energy is small and, hence, high-power densities can be achieved with low-power lasers, producing less heat and are cheaper than high-power versions of the same laser. Because flat top optics are very carefully calculated, one should keep in mind that the use of multiple laser lines is a feature that needs very careful planning and integration.

## 14. Conclusion

Analysis of single RNAs has proven its potential to help our understanding of regulatory processes in cells in greater detail than possible by ensemble measurements. Technology development in recent years has opened avenues into this research field by making the necessary equipment available to many laboratories. Here we provide tools, references, and background discussion that are needed before starting these kinds of experiments, both at the level of fixed and living cells. It becomes clear that light microscopy today has become an interdisciplinary research field that allows addressing questions from synthesis to decay of mRNA inside of cells with a temporal and spatial resolution unavailable before. Numbers of RNA molecules and distinct positions can be analyzed and experiments designed that allow looking at changes in these numbers and positions as a result of biological changes. In living cells, it becomes possible to investigate interaction times and mobility features of RNA. However, as with any new technology, this approach has its own features, which sometimes do not come naturally to the biologically oriented researchers. It is the goal of this chapter to address the more hidden aspects of single molecule microscopy while presenting the benefits of RNA research to the reader.

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## Appendix

The following presents a starting point for how to define the parameters necessary to generate meaningful data from single molecule approaches. Two major effects limit the number of single molecules that can be observed at the

same time: Resolution and mobility. Imaging is diffraction limited, which means the point spread function of the emission signal will be larger than the actual molecule by a factor given by the resolution of the microscope. (What you see in the image is much larger than the actual molecule you are observing because of distortions introduced by the imaging process.) To separate single molecules, the molecules have to be isolated well enough so that the individual diffraction limited signals can be evaluated. If individual molecules come too close to each other, they will not be resolvable. The lateral resolution of a light microscope is described in Eq. (27.1).

$$r_{x,y} = 0.61\lambda/\mathrm{N.A.} \tag{27.1}$$

Here $\lambda$ is the wavelength of the emitted light in nm and *N.A.* is the numerical aperture of the objective. As discussed earlier, fitting the signal precisely requires a sufficient spread of the signal over a couple of pixels (Thompson *et al.*, 2002). The fit becomes more stable if more than the absolute minimum of pixels is used. Practically, the resolution limit is too small to be used as minimum criteria for interparticle distances in this approach, because the localization precision is better the more isolated the signals are. The use of photoswitchable labels with techniques like PALM and STORM, temporal distances between signals allow construction of high-resolution images of labels that are in closer spatial proximity than the resolution limit (Betzig *et al.*, 2006; Hess *et al.*, 2006; Rust *et al.*, 2006). Although some groups use a small number of large pixels (Schmidt *et al.*, 1995), other groups use higher spatial oversampling (Thompson *et al.*, 2002; Yildiz and Selvin, 2005). To test the experimental procedure, test samples can be prepared with dyes or beads at different concentrations and then imaged in an immobilized state. Signals should show a full-width at half-maximum (FWHM) corresponding to the resolution of the optical system. The FWHM is a very descriptive measure. It is the width of the signal distribution at 50% of the intensity level. A Gaussian function is used to describe the signal distribution. Each axis can be fitted independently, that is, along the x and y coordinates of the image. The width of the fit is described by the standard deviation ($\sigma$):

$$y = y_0 + A\ \exp(-0.5((x - x_c)/\sigma)^2) \tag{27.2}$$

The intensity ($y$) is a function of the position ($x$), the parameter $A$, which scales for the Area under the fit curve, $x_c$ indicating the center position of the distribution and $y_0$, which is related to background offsets in the image if no flat fielding is used in prior image processing. For experimental data, it is noteworthy that the FWHM can be calculated from the fit data with the standard deviation ($\sigma$) of the fit accordingly.

$$\mathrm{FWHM} = 2(2\ln(2))^{1/2}\sigma \tag{27.3}$$

This is also valuable data to measure the localization precision and its dependence on the signal-to-noise ratio.

Once the molecules start moving, their mobility will be limiting for the number of observable molecules per image. The limit for being able to track particles is related to the question of how to make sure a particle observed in one frame is coming from the same molecule in the next frame. If molecules have a high chance to exchange for each other (when there are many mobile molecules close together), tracking will become arbitrary. Axial mobility in the z-axis will also show this problem. The relation between the diffusion coefficient ($D$) and the jump distance ($r$) of a molecule is given by:

$$D = \langle r^2_{x,y} \rangle / (2f\Delta t_{lag}) \tag{27.4}$$

The parameter $f$ scales Eq. (27.4) for the dimensions in which movement is observed. The time between two frames is indicated by $\Delta t_{lag}$. Imaging is normally two-dimensional, because the detector is planar, and although the particle moves in 3D, only the 2D projection of the movement is seen on the detector. The assumption behind this is that the molecule experiences an isotropic environment. Here a movement in any direction can be written as a vector with x, y, and z components. The assumed probability distribution for each component (x, y, and z) is equal, and, hence, any of these components represents the mobility sufficiently. Measuring the jump from one frame to the next will give a uniform time $t_{lag}$ that equals the frame rate of detection. Leaving one frame out will double the lag time, leaving two frames out will triple it. Linearity over such time scales indicates diffusion, and divergence from linearity can be related to corralled movement or active transport (Saxton and Jacobson, 1997).

The diffusion constant of a sphere can be estimated as a function of the temperature ($T$ in [K]), the Boltzmann constant ($k_B$), the radius of the sphere ($r$) and the viscosity of the environment ($\eta$) by:

$$D = k_B T / (2f\pi\eta r) \tag{27.5}$$

The dimensionality of movement that normally occurs in all three dimensions is represented by the $f$ factor. Here f is three because mobility is in 3D. In Eq. (27.4) it will still be two, because the observation is two dimensional. For a detailed discussion on how to deal with other geometric shapes, see Berg (1993).

By use of the molecular mass ($m$) of a protein and a mass density ($\rho$) of 1.2 [g $cm^{-3}$] (Andersson and Hovmoller, 1998), the radius can be estimated by:

$$r = ((3m/N_A)/(4\pi\rho))^{1/3} \tag{27.6}$$

With this small theoretical framework, it becomes possible to estimate the influence of mobility on the imaging conditions. The diffusion coefficient is a parameter that shows scattering. The mean square $\langle x^2 \rangle$ of the jump distance in Eq. (27.4) clearly indicates this. It becomes even more obvious when the probability function for a two-dimensional diffusion process is used. This function ($P(r,t)$) describes the jump distance ($r$) at time ($t$):

$$P(r,t) = 1/(4Dt) * \exp(r^2/(4Dt)) * r \tag{27.7}$$

It is used to evaluate jump distance histograms and can be written to account for multiple mobility classes:

$$P(r,t) = \sum\nolimits_i N_i/(4D_i t) * \exp(r^2/(4D_i t)) * r \tag{27.8}$$

More descriptively, this means that the mean jump distance is the center value of a distribution of jump lengths, and longer jumps will happen. Determining how many mobile molecules can be observed at the same time is related to this distribution and by the probability that molecules can exchange places with each other between two frames and, hence, cannot be identified individually. Because exchange can happen within the x, y plane but also along the optical axis (z), this is a profound limitation that reduces the number of observable molecules dramatically as the mobility increases. For tracking, one can define a criteria of minimum distances between two molecules (e.g., $3\sigma$ of the width of the jump distance distribution) and terminate tracking if molecules are identified below this distance to each other. (Imagine a circle with the radius of this value around each molecule; if circles from different molecules overlap in one frame, tracking is terminated for the next frame.) For identification of the molecule in the next frame, this circle can be used to search for the molecule. If more than one molecule is found, one needs to either discard the track or use other criteria to discriminate the positions into individual tracks. Nearest neighbor criteria are widely used (Saxton, 1997; Thompson *et al.*, 2002), but lately other criteria have emerged in the field, for example, with signal correlation or flow conservation (Gennerich and Schild, 2005; Vallotton *et al.*, 2003; 2005). On the basis of the assumptions made, each criterion will present a bias to the data. For example, the use of the nearest neighbor will select for short jump distances and underestimate mobility.

## Experimental Controls

Immobilized dyes or beads are standard tools for calibrating imaging systems. Imaging of immobilized beads allows users to define the localization precision and drifts of the microscope. Imaging beads or dyes for a

few hundred frames is performed for the following two controls, calculating *average position* and *standard deviation* of the signal. In these control images, imaging parameters should be identical to those used in the experimental conditions and must be performed at signal-to-noise ratios (SNR) similar to the experimental levels. To calibrate localization precision for different SNRs, only the intensity of the excitation light needs to be regulated; frame rate and other parameters should remain constant. The standard deviation of the position of the beads is the localization precision at this SNR. To check for physical drift of the microscope stage, the bead should be imaged for a period at least the duration of a normal experiment, longer if possible. For this, one can take fewer images over longer times at high SNRs, resulting in a time stack with low bleaching and more precisely localized single bead signals over time. The position of these beads can be plotted as a function of time, and this directly reflects the positional stability of the microscope. Because most microscope stages show some drift, it will either be necessary to carefully choose observation times for which drifts are smaller than the localization precision or for which the total precision is still acceptable. Alternately, internal controls, like beads or quantum dots (Qdots), could be imaged simultaneously during the experiment to allow image correction after acquisition. Qdots can present large Stoke shifts. This is an advantage that can be used to excite them with the same wavelength used for imaging and detect them concurrently with a different channel than the fluorophore being studied. The use of an immobilized signal on a glass surface to calibrate intensities to be observed in living cells will represent an upper limit for precision. Changes in the refractive index within the cell and light scattering on membranes affects the signals obtained and affects the precision. Calibrating the test slide signals at different intensities and, hence, different SNRs, compensates partially for these effects.

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