CHAPTER SEVENTEEN

Imaging Single mRNA Molecules in Yeast

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Contents

Abstract

Yeast cells in an isogenic population do not all display the same phenotypes. To study such variation within a population of cells, we need to perform measurements on each individual cell instead of measurements that average out the behavior of a cell over the entire population. Here, we provide the basic concepts and a step-by-step protocol for a recently developed technique enabling one such measurement: fluorescence *in situ* hybridization that renders single mRNA molecule visible in individual fixed cells.

1. Introduction

Within an isogenic population of yeast cells, the behavior of any individual cell can differ markedly from the average behavior of the population (Raj and van Oudenaarden, 2008). For example, it has been shown

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that random partitioning of proteins during cell division leads to variability in the number of proteins in individual cells (Rosenfeld *et al.*, 2005), while random bursts of transcription results in variability in number of mRNAs (Chubb *et al.*, 2006; Golding *et al.*, 2005; Raj *et al.*, 2006). These are just a few examples that highlight the importance of studying the behavior of a single cell rather than that of the whole population. One primary tool for studying the behavior of a single cell is the fluorescent protein such as GFP (green fluorescent protein). The most straightforward application of a fluorescent protein is to have it either driven by the promoter of interest or fused to the protein of interest to study variability in gene expression. Yet while the use of fluorescent proteins has certainly been pivotal in monitoring gene expression, fluorescent proteins suffer from a number of limitations. One such limitation is their low sensitivity: fluorescence from GFP and its variants is typically undetectable at the small number of molecules involved in studying gene expression. In yeast, fluorescence from GFP is typically detectable only when many hundreds of GFPs are present in a cell; the abundance of many transcription factors, for example, falls below this limit. Since the effects of expression variability are magnified when the number of molecules is low, the sensitivity limitation may preclude effective study of these processes. Another issue is that it is difficult to quantify the exact number of fluorescent proteins in individual cells because it is difficult to measure the amount of fluorescence emitted by a single GFP molecule. In addition, the slow decay time of fluorescent proteins (due to their relatively high stability) means that fluorescence is only diluted by cell division but not through other degradation mechanisms. This prevents observation of rapidly varying changes in gene activation, effectively averaging temporal fluctuations.

While having a fluorescent protein expressed by the promoter of interest or fused to a protein of interest suffers from a number of setbacks, other applications of the fluorescent protein led to powerful techniques enabling the detection of a single mRNA molecule in a single cell. mRNA of a given gene in a single cell has been difficult to detect in the past because each cell has very small copy numbers of it at any one time. One such technique is the MS2 mRNA detection scheme (Beach *et al.*, 1999; Bertrand *et al.*, 1998). One way to implement this technique is to engineer a gene so that its mRNA contains 96 copies of a particular RNA hairpin in its untranslated region. These hairpins then tightly bind to a coat protein of the bacteriophage MS2. Therefore, by also having a gene expressing the MS2 coat protein fused to GFP in the cell, a single mRNA with the 96 copies of RNA hairpin will now emit high enough fluorescence to be resolved as a single diffraction-limited spot under a fluorescence microscope. This method can help measure the transcription of a gene in real-time in a single-cell, as was done in *Escherichia coli* (Golding *et al.*, 2005). Despite the vast improvement in resolution the MS2 method provides over conventional methods using

GFP and its variants, it has a disadvantage in that mRNAs tend to aggregate together and that the regulation of the endogenous mRNA may change (thus one monitors this altered regulation rather than the endogenous one) because it has now been engineered to have the long artificial sequence for hairpin formation.

In this chapter, we describe fluorescence *in situ* hybridization (FISH) method (Gall, 1968; Levsky and Singer, 2003) for detecting single endogenous mRNA molecules in individual yeast cells (Raj *et al.*, 2008). Since the target gene sequence does not have to be modified to use this method, it bypasses the aforementioned problems associated with engineering the mRNA to have hairpin forming sequences in the MS2 mRNA detection scheme. It is also highly sensitive and allows for the counting of mRNA molecules in single cells, thus obviating many of the issues associated with using GFP as either a fusion to a protein of interest or driven by a promoter of interest mentioned before. In this method, we utilize a large collection (at least 30) of oligonucleotides, each labeled with a single fluorophore, that binds along the length of the target mRNA (Fig. 17.1A). The binding of so many fluorophores to a single mRNA results in a signal bright enough to be detectable with a microscope as a diffraction-limited spot. The method we describe is a modification of the RNA FISH method described by Singer and coworkers (Femino *et al.*, 1998), in which the authors use a smaller number (~5) of longer oligonucleotides (~50 bp), each of which contains up to five fluorophores (Fig. 17.1B). While that method has been used successfully to count mRNAs in single cells (Long *et al.*, 1997; Maamar *et al.*, 2007; Sindelar and Jaklevic, 1995; Zenklusen *et al.*, 2007), it has not been widely adopted. This may be due to the difficulties and costs associated

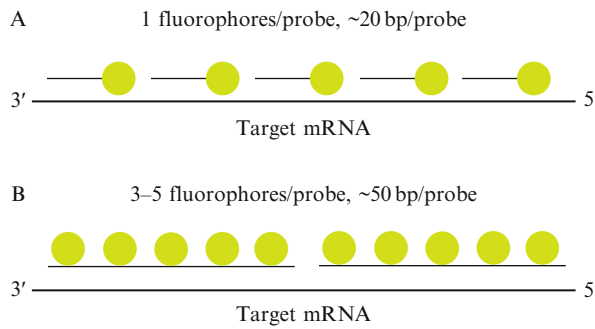

Figure 17.1 Comparison between two *in situ* hybridization methods for imaging a single mRNA molecule. (A) Method of Raj *et al.* (2008) involves about 30 or more singly labeled probes, each about 20 bases long, that bind along the stretch of a target mRNA molecule. (B) Method of Femino *et al.* (1998) involves multiple fluorophores (between 3 and 5) coupled to a single oligonucleotide probe of about 50 bases long that bind along the stretch of a target mRNA molecule.

with synthesizing and purifying several oligonucleotides with the internal modifications required to label those oligonucleotides with multiple fluorophores. Another potential issue is self-quenching between tightly spaced fluorophores. We anticipate that the simplicity of the method described herein will allow many researchers to utilize single-molecule RNA FISH in their own studies.

2. RNA FISH Protocol

A brief overview of our method is as follows. A set of short (between 17 and 22 bases long) oligonucleotide probes that bind to a desired target mRNA are designed and are coupled to a fluorophore (such that one oligonucleotide probe is bound to a single fluorophore) with desired spectral properties. After fixing the yeast cells, these probes are hybridized to the target mRNA molecule. This results in multiple (typically about 48) singly labeled probes bound to a single mRNA molecule. In turn, the mRNA molecule can give off enough fluorescence to be detected as a diffraction-limited spot using a standard fluorescent microscope. Below we describe a step-by-step procedure for implementing RNA FISH in *Saccharomyces cerevisiae*.

2.1. Designing oligonucleotides

The first step is the design of a collection of oligonucleotide probes that together are complimentary to a large part of the open read frame of the target mRNA (one can also utilize the untranslated regions of the mRNA, if necessary). Each probe is between 17 and 22 bases long and we have generally found that 30 or more such probes are sufficient to give a detectable signal. We have also found that our signals are sometimes clearer when the GC content of each probe is close to 45%. We also leave a minimum of two bases as a spacer between two adjacent probes that cover the mRNA, although it is possible that one can relax this requirement without any adverse effects. A program that facilitates the designing of probes meeting the constraints mentioned above is available freely at http://www.singlemoleculefish.com. Sometimes it is not possible to design probes that meet all the constraints mentioned above, and these criteria should not be viewed as absolutes, but more as guidelines we try to adhere to when possible. After designing the probes, we order them from companies with parallel synthesis capabilities (we use BioSearch Technologies based in Novato, CA, USA) with 3′-amine modifications. Since the synthesis typically results in a much larger number of oligonucleotides than are necessary, one should have them synthesized on the smallest possible

scale (we typically have them synthesized on the 10 nmol (delivered) scale). The 3′-amine then serves as a reactive group for the succinomidyl-ester coupling of the fluorophore described in Section 2.2.

2.2. Coupling fluorophores to oligonucleotides

The next step is the attachment of a fluorophore with desired spectral properties to the commercially synthesized oligonucleotides (we will describe which fluorophores we use in Section 2.2.1.) We do this by pooling the oligonucleotides and coupling them *en masse*, thus reducing the labor involved. In all the steps we describe below, we use RNase free water (Ambion) to prepare our solutions and use filtered pipette tips to prevent aerosol contaminations.

Procedure:

1. From the commercially synthesized set of oligonucleotides, each at a concentration of 100 μM in RNase free water (we find this is a practical starting concentration to work with), pipette around 1 nmol/10 μl of each oligonucleotide probe into a single microcentrifuge tube (i.e., if there are 48 probes, then 1 nmol of each of the 48 probe solutions should be combined into a single tube with a final volume of 480 μl).
2. Add 0.11 volumes (v/v) of 1 M sodium bicarbonate (prepared with RNase free water) to this probe mixture, resulting in a final sodium bicarbonate concentration of 0.1 M. If the total volume of the mixture at this stage is less than 0.3 ml, add enough 0.1 M sodium bicarbonate to bring the final volume of the mixture to 0.3 ml.
3. Dissolve roughly 0.2 mg of the desired fluorophore (functionalized with a succinimidyl ester group) separately into a tube containing 50 μl of 0.1 M sodium bicarbonate. If using tetramethylrhodamine (TMR), first dissolve it in about 5 μl of dimethyl sulfoxide (DMSO) and then add 50 μl of 0.1 M sodium bicarbonate to it. This is because TMR does not readily dissolve in aqueous solutions.
4. Add the dissolved fluorophore to the 0.3 ml of probe mixture, vortex, and cover this tube in aluminum foil to prevent photobleaching from unwanted exposure to ambient light. Leave the tube in the dark overnight.
5. Next day, precipitate the probes out of solution by adding 12% (v/v) of sodium acetate at pH 5.2 followed by 2.5 volumes of ethanol (95% or 100%).
6. Place the tube at −70 °C for at least 1 h, then spin the sample down at 16,000 rpm for at least 15 min at 4 °C.
7. A small colored pellet should have collected at the bottom of the tube at this stage. This pellet contains both the coupled and uncoupled oligonucleotides. The vast majority of the uncoupled fluorophore, however,

remains in the supernatant, and so aspirate as much of this supernatant away as possible without disturbing the pellet (one should take care to aspirate soon after removal from the centrifuge, since oligonucleotides have a tendency to redissolve rapidly at room temperature.

Note: Many precipitation protocols now call for another washing step in 70% ethanol. We have found this step unnecessary.

8. The pellet is stable and can be stored in -20 °C for up to 1 year. This concludes the coupling step.

2.2.1. Choice of fluorophore and appropriate filter sets

In order to perform imaging of multiple different RNA species at the same time, one needs to select fluorophores with excitation and emission properties that can be distinguished by appropriately chosen bandpass filters; otherwise, the signal from one channel may potentially bleed into another channel. We describe here the fluorophore and filter set combination that we use for our microscopy. Other combinations are no doubt feasible as well.

The fluorophores we utilize are TMR, Alexa 594, and Cy5. TMR has proven to be exceptionally photostable in our hands, and its excitation maximum of 550 nm aligns nicely with the excitation maxima of mercury and metal-halide light sources. Alexa 594 is also quite photostable, and while its spectral properties are similar to those of TMR (absorption at 594 nm), we are able to distinguish its presence using appropriate filters. The third fluorophore we use is Cy5, which is rather bright and is spectrally separated from the other two fluorophores (Cy5 absorbs at 650 nm). Cy5 does, however, suffer from photobleaching effects, thus requiring the use of a glucose oxidase oxygen scavenging system to make imaging feasible. We have not tried any dyes that are further redshifted than Cy5. However, we have experimented with Alexa 488, which absorbs at a lower wavelength than TMR. While we were sometimes able to detect signals, the higher cellular background at these lower wavelengths lead to weaker signals, so we generally avoid the use of fluorophores bluer than TMR.

The filter combinations we use are typical bandpass filter and dichroic sets mounted in cubes that the microscope can place in the fluorescence light path. For TMR, we use a standard XF204 filter from Omega Optical. For Alexa 594, we use a custom filter from Omega Optical with a 590DF10 excitation filter, a 610DRLP dichroic, and a 630DF30 emission filter. For Cy5, we use the 41023 filter from chroma, which is designed for Cy5.5. It is likely that a filter more appropriate for Cy5 would work even better. These filters do a good job of preventing any signals from one fluorophore from being detected in another channel (Raj *et al.*, 2008). Sometimes a very bright Alexa 594 signal can bleed somewhat into the TMR channel

(we estimate the bleedthrough to be about 10%) but practically this bleedthrough is impossible to detect owing to the low signal intensities of the mRNA spots.

2.3. Purification of probes using HPLC

We now describe a purification procedure for separating the coupled oligonucleotides from the uncoupled oligonucleotides. We purify the coupled oligonucleotides using HPLC (high-performance liquid chromatography): the addition of the fluorophore makes the normally hydrophilic oligonucleotide significantly more hydrophobic, allowing for separation by chromatography. The HPLC should be equipped with a dual wavelength detector for a simultaneous measurement of absorption by DNA (at 260 nm) and fluorophore (depends on the fluorophore: e.g., 555 nm for TMR and 594 nm for Alexa 594). In our lab, we have used an Agilent 1090 equipped with Chemstation software and a C18 column suitable for oligonucleotide purification (218TP104). The two buffers used for HPLC are: 0.1 *M* triethylammonium acetate (''Buffer A'') and acetonitrile (''Buffer B'').

Procedure:

1. Before running the purification program on the HPLC, equilibrate the column by flowing 93% Buffer A/7% Buffer B through for about 10 min; if the column is not equilibrated, then the oligonucleotides will simply flow straight through without any separation.
2. Resuspend the oligonucleotide pellet in an appropriate volume of water (we use 115 μl) and then inject this into the HPLC inlet.
3. Run an HPLC program in which the percentage of Buffer A varies from 7% to 30% over the course of about 45 min with a flow rate of 1 ml/min. During the execution of the program, carefully monitor the two absorption curves, one for DNA (at 260 nm) and the other for the coupled fluorophore (e.g., 555 nm for TMR and 594 nm for Alexa 594). Generally speaking, one will observe two broad peaks over time. The first peak, containing the more hydrophilic material, consists of the uncoupled oligonucleotides and will only exhibit absorption in the 260 nm channel (Fig. 17.2A). This peak may appear relatively ragged due to the presence of multiple oligonucleotides, each of which has a slightly different retention time in the HPLC. The second peak, often narrower than the first, will appear some time after the first peak and contains the coupled oligonucleotides; thus, it will show absorption in both the 260 nm and the fluorescent (e.g., 555 nm) channels (Fig. 17.2B). The duration of time between the first and second peaks varies depending on the hydrophobicity of the fluorophore; we have found that oligonucleotides coupled to Cy5 have a long

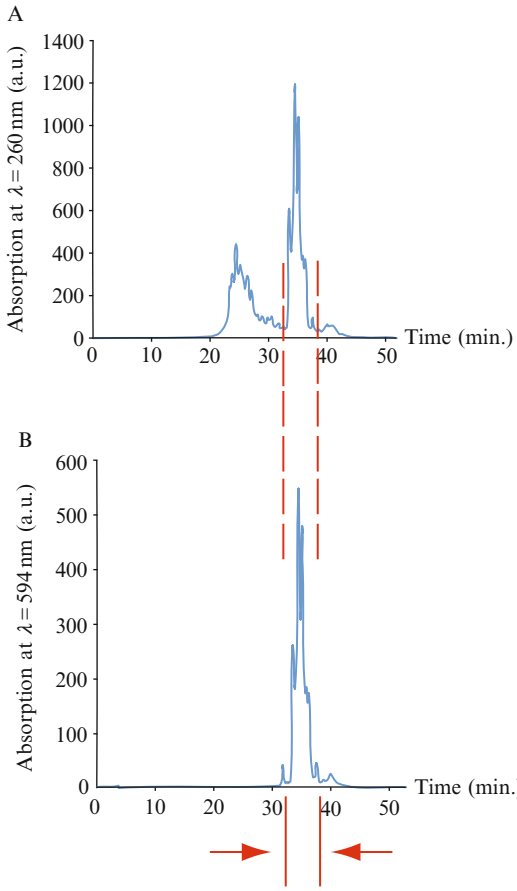

Figure 17.2 Chromatographs obtained during the HPLC purification of oligonucleotides coupled to the fluorophore (Alexa 594) from uncoupled oligonucleotides. (A) Absorption (at 260 nm, for DNA) curve as a function of time monitored during purification of probes coupled to Alexa 594 using HPLC. The first peak that appears between 20 and 30 min in this channel correspond to oligonucleotide probes that do not have Alexa 594 coupled to them. Eluate is not collected for the duration of this peak. (B) Absorption (at 594 nm, for Alexa 594) curve as a function of time. Both absorption curves (A) and (B) are obtained simultaneously for the duration of the HPLC run. Only one distinct peak appears in this channel, representing absorption by probes with Alexa 594 successfully coupled to them. This peak coincides with the second peak in the 260 nm channel shown in (A). Eluate is collected for the entire duration of this peak in the 594 nm channel.

retention time of almost 20 min after the first peak, whereas TMR and Alexa 594 result in shorter retention shifts (Fig. 17.2B).

4. Collect the contents of this peak (in the flurophore absorption channel) manually into clean, RNase free tubes. It is important to collect all the

solution that is coming out of the outlet, starting from the beginning of the left shoulder of this second peak and stopping the collection just at the tail-end of the right shoulder of this second peak (Fig. 17.2B), because the different coupled oligonucleotides will have slightly different retention times; do not just "collect the peak." This collection typically lasts around 3–7 min in our experience. With the volumes we mentioned for our HPLC setup above, we typically collect between 5 and 14 ml in this step with 0.5 ml/tube. The program we use then typically flows 70% Buffer B through the column for about 10 min. This step will "strip" the column of any impurities that may have stuck to the column and is especially important if you plan to purify additional probes. Be sure, however, to allow sufficient time for the column to reequilibrate to 7% B/93% A before injecting another sample.

5. After collecting the solution of coupled probes, dry the collection in a SpeedVac rated for acetonitrile until the liquid is fully evaporated (about 3–5 h). It is important to keep light out of the SpeedVac to avoid photobleaching of dyes, especially for highly photolabile cyanine dyes such as Cy3 and especially Cy5.
6. Resuspend the contents in a total of 50–100 μl of TE (10 m*M* Tris with HCl to adjust pH, 1 m*M* EDTA, Ambion) at pH 8.0. This final suspension solution is now the "probe stock."
7. From the "probe stock," create dilutions of 1:10, 1:20, 1:50, and 1:100 in TE to make "working stocks." This dilution series is used to determine which concentration of probes yields the best signals for RNA FISH.
8. Store these probes in dark at -20 °C until sample is ready to be prepared. We found that the probes can be stored for years in this way.

2.4. Fixing *S. cerevisiae*

Having isolated the coupled probes, it is now time to fix the yeast cells so that these probes can be hybridized to their target mRNAs in these cells. In the following procedure, we have adopted the procedure for fixing *S. cerevisiae* from Long *et al.* (1995).

Procedure:

1. Grow the yeast cells to an OD of around 0.1–0.2 (corresponding to about 1–2 $\times$ 10^6 cells/ml) in a 45-ml volume of minimal media with appropriate supplements (depending on the auxotroph) in a batch shaker at 30 °C (we use 225 rpm).
2. Add 5 ml of 37% formaldehyde (i.e., 100% formalin) directly to the growth media containing the cells and let it sit for 45 min at room temperature to fix the cells. One should take safety precautions when using the carcinogen, formaldehyde (i.e., use chemical fume hood, gloves, and long-sleeved protective clothing).

3. Concentrate the cells in this 50 ml into a single microcentrifuge tube. We found that one way to concentrate the cells was to run the above 50 ml mixture through a vacuum filter (with a filter paper having 0.2 μm pores: VWR vacuum filtration system "PES 0.2 μm") once, then shake the filter paper into an RNase free water. Alternately, one may simply centrifuge the content at 2300 rpm for about 5 min and then resuspend in 1 ml Buffer B to transfer to a microcentrifuge tube.
4. Wash these concentrated cells in the microcentrifuge tube twice with 1 ml ice-cold Buffer B (Long *et al.*, 1995).
5. Add 1 ml of spheroplasting buffer (from a stock made by adding 100 μl of 200 m*M* vanadyl-ribonucleoside complex to 10 ml Buffer B), and transfer the mixture to a new RNase free microcentrifuge tube.
6. Add 1 μl of zymolyase and incubate at 30 °C for about 15 min; this spheroplasting step removes the cell wall and is important for probe penetration.
7. Wash the solution twice with 1 ml ice-cold Buffer B, with centrifuging the content at 2000 rpm for 2 min in between.
8. Add 1 ml of 70% ethanol (diluted in RNase free water) to the cells and leave them for an hour or even overnight at 4 °C.

The yeast cells have now been fixed and are ready for hybridization. These cells can be stored in ethanol for up to a week after fixation and perhaps even longer.

2.5. Hybridizing probes to target mRNA

The hybridization step contains three key parameters that may be varied to optimize the FISH signal. These are the temperature at which hybridization takes place, the concentration of formamide used in the hybridization and wash, and the concentration of the probe. The first two parameters essentially set the stringency of the hybridization; that is, the higher the temperature or the concentration of formamide, the lower the likelihood of nonspecific binding of the probes. We usually elect to adjust the formamide concentration rather than temperature and thus perform all FISHs at 30 °C. Typically, we have found that hybridization and wash buffers containing 10% formamide work quite nicely for most probes, yielding a fairly low background while also producing clear particulate signals. However, when the GC content of the probes is relatively high (>55%), we have found that we sometimes have to employ formamide concentrations up to 20% or sometimes higher. However, care must be taken in these instances, since the use of higher formamide concentrations can sometimes lead to a greatly diminished signal. Generally, we try to obtain signals at a standard concentration of formamide, because this greatly facilitates the simultaneous

detection of multiple mRNAs: if the hybridization conditions are the same, multiplex detection is simply a matter of mix and match.

The concentration of probe used is also very important in obtaining clear, low background signals. Typically, the optimal probe concentration must be found empirically, but we have found that concentrations can vary over roughly an order of magnitude and still produce satisfactory results. We typically start by using a 1:1000, 1:2500, and 1:5000 dilution of the original stock into hybridization buffer. One of these concentrations will usually yield good signals, but sometimes one must use drastically lower concentrations (100-fold lower) in order to obtain signals.

2.5.1. Preparation of hybridization and wash buffers

The following procedure describes preparation of 10 ml of hybridization buffer with the desired formamide concentration. Be sure to adjust the volumes appropriately if you are preparing a different total volume of hybridization buffer.

Procedure:

1. Dissolve 1 g of high molecular weight dextran sulfate (>50,000) in approximately 5 ml of nuclear free water. Depending on the particular preparation of dextran sulfate used, the powder may dissolve quite rapidly with a bit of vortexing or may require rocking for several hours at room temperature. In the end, the solution should be clear and fairly viscous, although some preparations are far less viscous but still appear to work.
2. Add 10 mg of *E. coli* tRNA (Sigma, 83854), vortexing to dissolve.
3. Add 1 ml of 20× SSC (RNase free, Ambion), 40 μl (to get 0.02% in 10 ml) of RNase free BSA (stock is 50 mg/ml = 5% solution from Ambion, AM261), 100 μl of 200 m*M* vanadyl-ribonucleoside complex (NEB S1402S), formamide to the desired concentration (10–30%), and then water to a final volume of 10 ml. When using formamide, one must first warm the solution to room temperature before opening to avoid oxidation; also, care must be taken when using formamide (i.e., use in the hood, wear protection, etc.) because it is a suspected carcinogen and teratogen and is readily absorbed through the skin.
4. Once the solution is thoroughly mixed, filter the buffers into small aliquots; this removes any potential clumps that can yield a spotty background. We simply filter the solution in 500 μl aliquots using cartridge filters from Ambion.
5. Store the solution at −20 °C for later use; solution is typically good for several months to a year.
6. Prepare the wash buffer by combining 5 ml of 20× SSC (Ambion), 5 ml of formamide (to final concentration of 10% (v/v); this is adjusted if the hybridization buffer has a different formamide concentration), and 40 ml of RNase free water (Ambion) into one solution.

2.5.2. Hybridizing probes to yeast cells in solution

Procedure:

1. Warm the hybridization solution to room temperature before opening its cap to prevent oxidation of the formamide.
2. Add 1–3 μl of desired concentration of probes to 100 μl of the hybridization buffer. To determine what the desired concentration of probes is, we initially perform hybridizations with four dilutions of probes: 1:10, 1:20, 1:50, and 1:100 (mentioned in Section 2.3), and see which dilution gives the clearest signal.
3. Centrifuge the fixed sample and aspirate away the ethanol, then resuspend the fixed cells in a 1-ml wash buffer containing the same formamide concentration as the hybridization buffer.
4. Let the resuspension stand for about 2–5 min at room temperature.
5. Centrifuge the sample and aspirate the wash buffer. Then add the hybridization solution.
6. Incubate the sample overnight in the dark at 30 °C.
7. Next morning, add 1 ml of wash buffer to this sample, vortex, centrifuge, then aspirate away the supernatant.
8. Resuspend in 1 ml of wash buffer, then incubate in 30 °C for 30 min.
9. Repeat the wash in another 1 ml of wash buffer for another 30 min at 30 °C, this time adding 1 μl of 5 mg/ml DAPI for a nuclear stain.

10A. *If using photostable fluorophores such as TMR or Alexa 594*: then there is no need to add the GLOX solution. Just resuspend the sample in an appropriate volume (larger than 0.1 ml) of 2× SSC and proceed to imaging.

10B. *If using a highly photolabile fluorophore such as Cy5*: resuspend the fixed cells in the GLOX buffer (used as an oxygen-scavenger that removes oxygen from the medium to prevent light-initiated fluorophore destroying-reactions; see Section 2.5.3) without the enzymes and incubate it for about 2 min for equilibration (see Section 2.5.3 for details). Then centrifuge, aspirate away the buffer and resuspend the cells in a 100-μl of GLOX buffer with the enzymes (glucose oxidase and catalase). These cells are now ready to be imaged.

We found that our samples (either with or without the antibleach solution) can be kept at 4 °C for a day's worth of imaging. Keeping the samples at 4 °C prevents the probe-target hybrids from dissociating and thus degrading the signals.

2.5.3. Preparation of antibleach solution and enzymes ("GLOX solution")

During imaging, we typically take several vertical stacks ("z-stacks") of images through a cell in a field of view, causing a hybridized fluorophore in a fixed cell to be excited by intense light several times. More importantly,

when more than one type of fluorophore is used for imaging two or three species of mRNA, such z-stacks must be repeated to excite each of the different fluorophores, leading to even more exposure of the fluorophores. In our experience, only TMR and Alexa 594 could withstand such repeated excitations, whereas Cy5 signal would rapidly degrade due to its especially high rate of photobleaching. To decrease the photolability of Cy5, we used an oxygen-scavenging system consisting of catalase, glucose oxidase, and glucose (GLOX solution) that is slightly modified from that used by Yildiz *et al.* (2003). This GLOX solution acts as an oxygen-scavenger that removes oxygen from the medium. Since the light-initiated reactions that destroy fluorophores require oxygen, the GLOX buffer thus prohibits these reactions from taking place. Indeed, we found that Cy5 was able to withstand nearly 10 times more exposure with the GLOX solution than without it. The following is a procedure for preparing the GLOX solution.

Procedure:

1. Mix together 0.85 ml of RNase free water with 100 μl of 20× SSC, 40 μl of 10% glucose, and 5 μl of 2 *M* Tris–Cl (pH 8.0). This is the GLOX buffer (without glucose oxidase and catalase).
2. Vortex the mixture, and then aliquot 100 μl of it into another tube.
3. To this 100 μl aliquot of GLOX buffer (glucose–oxygen-scavenging solution without enzymes), add 1 μl of glucose oxidase (from 3.7 mg/ml stock, dissolved in 50 m*M* sodium acetate, pH 5.2, Sigma) and 1 μl of catalase (Sigma). Before pipetting the catalase, vortex it a bit, since the catalase is kept in suspension (also, care should be taken when handling the catalase, since it has a tendency to get contaminated). This 100 μl will be referred to as "GLOX solution with enzymes." The GLOX solution without the enzyme will later be used as an equilibration buffer.

2.5.4. Imaging samples using fluorescent microscope

The fixed cells with probes properly hybridized are now ready for imaging. Our microscopy system is relatively standard: we use a Nikon TE2000 inverted widefield epifluorescence microscope. It is important to use a fairly bright light source. For instance, a standard mercury lamp will suffice, although the newer metal-halide light sources (e.g., Lumen 200 from Prior) tend to produce a more intense and uniform illumination. Another important factor is the camera. It is important to use a cooled CCD camera that is optimized for low-light imaging rather than acquisition speed; we use a Pixis camera from Roper. Also, the camera should have a pixel size of 13 μm or less. We should point out that the signals from the newer EMCCD cameras are no better than these more standard (and cheaper) cooled CCD cameras. We typically use a 100× DIC objective. If one is interested in imaging with Cy5, one must be sure that the objective has sufficient light transmission at those longer wavelengths; this can sometimes

require an IR coating. When mounting the cells, it is important to make sure that one uses #1 coverglass (18 mm × 18 mm, 1 ounce) and that the yeast are directly on the coverglass: do not adhere the yeast to the slide and then cover with coverglass. One can enhance the adherence of the yeast to the coverglass by coating the coverglass with poly-L-lysine (put fresh 1 mg/ml poly-L-lysine solution on the coverglass for 20 min, then suction off) or concanavalin A. It is also important to use #1 coverglass: we have found that even though most objectives are corrected for #1.5 coverglass, the mRNA spots are usually fuzzier and less distinct when imaged through #1.5 coverglass.

There are two somewhat standard procedures often employed during fluorescence microscopy that we have found interferes with our single mRNA signals. One of these is the use of commercial antifade mounting solutions, which tend to introduce a large background while also decreasing the fluorescent signals from target mRNAs. We recommend instead using the custom made GLOX solution or 2× SSC for imaging, being careful not to let the sample dry out. We also discourage using the standard practice of using a nail polish to seal the sample, as it introduces a background autofluorescence in the red channels that interferes with fluorescence from mRNA.

2.6. Image processing: Detecting diffraction-limited mRNA spot

We have devised an algorithm that automates some fraction of the work involved in analyzing images obtained from the samples (Raj *et al.*, 2008). The first step in our algorithm is applying a three-dimensional linear filter that is approximately a Gaussian convolved with a Laplacian to remove the nonuniform background while enhancing the signals from individual mRNA particles, thus enhancing the signal-to-noise ratio (SNR) (Fig. 17.3B). The full width at half maximum of this Gaussian corresponds to the optimal bandwidth of our filter, and depends on the size of the observed particle. This width is a fit parameter that we empirically adjust to maximize the SNR. However, even after filtering the images, they will contain some noise that requires thresholding to remove. In order to make a principled choice of threshold, we sweep over a range of possible values of the threshold, and plot the number of mRNAs detected at each value (Fig. 17.3C). Here, a single mRNA is defined as a collection of localized pixels (in the series of z-stacks) that form a connected component (Fig. 17.3D). We then typically find a plateau in this plot of the number of mRNAs counted as a function of the value of the threshold (i.e., increasing the threshold does not change the number of mRNAs counted) as seen in Fig. 17.3C. This implies that the signals from mRNAs are well separated from the background noise rather than a smooth

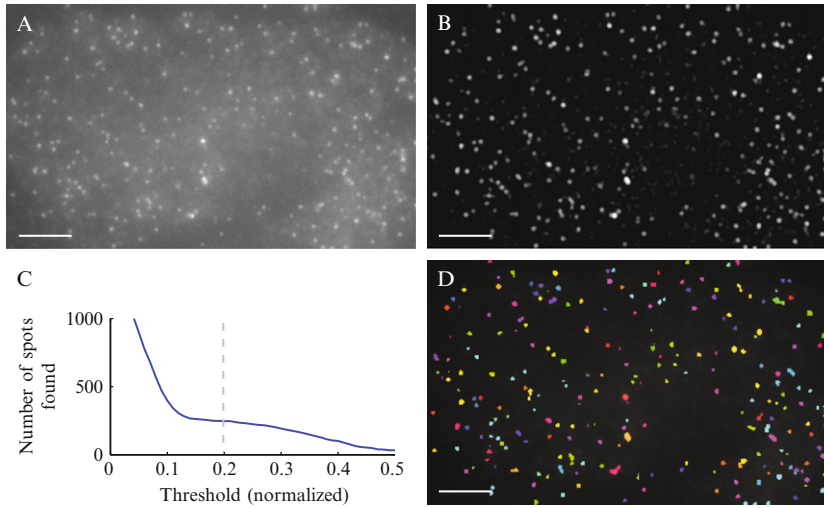

Figure 17.3 Example of mRNA spot detection algorithm applied to raw images of *FKBP5* mRNA particles in A549 cells induced with dexamethasone. (A) Raw image data showing *FKBP5* mRNA particles. (B) Upon applying a three-dimensional linear filter that is approximately a Gaussian convolved with a Laplacian to remove the nonuniform background while enhancing the signals from individual mRNA particles on the raw image shown in (A) the SNR is increased. (C) The number of spots counted as a function of the threshold value of the background after the application of the linear filter shows an existence of a plateau. This indicates a clear distinction between background fluorescence and actual mRNA spots. (D) Using the value of threshold shown as the gray line in (C), the raw image (A) has been transformed to an image in which each distinct computationally identified spot has been assigned a random color to facilitate visualization. Reprinted with permission from Raj *et al.* (2008).

"blending" in of the mRNA signals with the background noise. Indeed, the value of threshold chosen in this plateau range yielded mRNA counts nearly equal to the mRNA counts we obtained through an independent method in which we count by eye without the aid of automation. The software used for this purpose is available for download on *Nature method*'s supplementary information site for Raj *et al.* (2008). One can also make measurements based on mRNA spot intensity, although we feel that great care must be taken in these situations. One issue is that the intensity depends on how precisely focused the spot is, although this can be ameliorated by taking a large number of closely spaced fluorescent stacks. Another problem with computing total or mean intensity is that the boundary of the mRNA is hard to define, and the ultimate intensity measurement will depend heavily on this somewhat arbitrary choice. One way to skirt the issue is to use the maximum intensity within a given spot, since this is independent of the size of the spot.

3. Example: *STL1* mRNA Detection in Response to NaCl Shock

As an application of the FISH technique we just outlined, and we now show an example of this technique applied to *S. cerevisiae*. One mRNA of interest in yeast is that of the *STL1* gene, whose expression level dramatically increases when the cell is subjected to an osmotic shock (Rep *et al.*, 2000). One way to induce such a shock is by increasing the concentration of NaCl in the cell's growth medium. For this purpose, a strain based on the common laboratory strain BY4741 (Mat a, his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, YER118^{c}::kanMX^{R}) was grown to an OD of 0.56 ($\sim 0.7 \times 10^{7}$ cells/ml) in a 50-ml volume of complete supplemental media without histidine and uracil. We then shocked them osmotically by transferring the cells to a medium with 0.4 *M* NaCl and leaving them there for 10 min. We fixed these shocked cells along with their unshocked counterparts using the method we outlined before (5 ml of 37% formaldehyde was added directly to the medium for 45 min). We adopted the fixation and spheroplasting procedures were from Long *et al.* (1995), but with the exception that after spheroplasting, the cells were incubated in concanavalain A (0.1 mg/ml, Sigma) for about 2 h before letting them settle onto a coverglass with a chamber that was coated with concanavalin A overnight. We used concanavalin A because it helped the yeast cells stick to a cover glass, although as mentioned earlier, it is possible also to simply use poly-L-lysine coated coverglass without incubating the cells in concanavalin A. The resulting images of RNA FISH performed on unshocked and shocked cells can be seen in Fig. 17.4A and B, respectively. As seen in these figures, the RNA

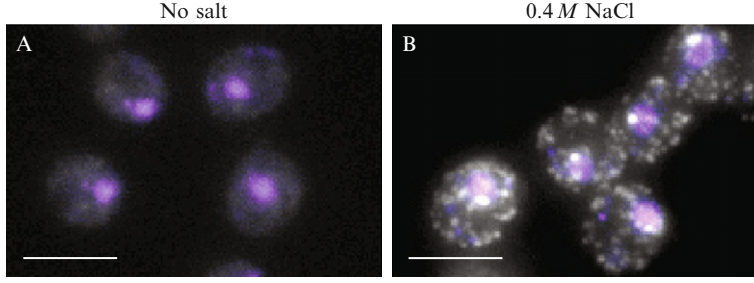

Figure 17.4 Single mRNA molecules imaged in *S. cerevisiae* using the fluorescence *in situ* hybridization method of Raj *et al.* (2008). Scale bars (white lines) indicate 5 μm. (A) *STL1* mRNA particles in yeast cells before being subjected to osmotic shock (0 *M* NaCl in the growth medium). (B) *STL1* mRNA particles in yeast cells 10 min after they have been growing in the presence of a high level of salt (0.4 *M* NaCl), thus inducing osmotic shock. DAPI was used to stain the nucleus of the cells shown in purple. The *STL1* gene expression increases dramatically after the osmotic shock. Reprinted with permission from Raj *et al.* (2008).

FISH technique of these workers (Raj *et al.*, 2008) allows one to not only resolve individual *STL1* mRNAs but also to extract spatial information on their whereabouts (helped by DAPI staining of the cell's nucleus). In addition, taking snapshots of *STL1* mRNAs at two different time points as shown in Fig. 17.4A and B illustrates how one can construct dynamics of the mRNA distribution in a population of cells by performing FISH on the cells at different time points.

4. Conclusions

Although we have limited our description of RNA FISH to just *S. cerevisiae*, this method has so far been applied to *E. coli*, *Caenorhabditis elegans*, *Drosiphila melanogaster*, and rat hippocampus neuronal cell cultures (Raj *et al.*, 2008). In fact, the protocol we described requires just a few adjustments in order to be applicable to these organisms. The method is likely to be applicable to other organisms as well. Studying how individual yeast cells behave through single cell measurements and using the distributions constructed through those measurements to look at how populations of cells behave remains a vital field of research today. We believe that the FISH method for visualizing a single mRNA molecule in yeast will play an important role in such endeavors.

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