CHAPTER TWENTY-SIX

# Analyzing mRNA Expression Using Single mRNA Resolution Fluorescent *In Situ* Hybridization

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## Contents



## Abstract

As the product of transcription and the blueprint for translation, mRNA is the main intermediate product of the gene expression pathway. The ability to accurately determine mRNA levels is, therefore, a major requirement when studying gene expression. mRNA is also a target of different regulatory steps, occurring in different subcellular compartments. To understand the different

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steps of gene expression regulation, it is therefore essential to analyze mRNA in the context of a single cell, maintaining spatial information. Here, we describe a stepwise protocol for fluorescent *in situ* hybridization (FISH) that allows detection of individual mRNAs in single yeast cells. This method allows quantitative analysis of mRNA expression in single cells, permitting "absolute" quantification by simply counting mRNAs. It further allows us to study many aspects of mRNA metabolism, from transcription to processing, localization, and mRNA degradation.

## 1. Introduction

The life cycle of an mRNA comprises many different steps. Starting with mRNA synthesis, mRNAs are processed, assembled into mRNPs, exported from the nucleus, sometimes localized, usually translated, and ultimately always degraded. These different steps along the gene expression pathway are tightly regulated and many are subjected to quality control steps that ensure their proper execution (Houseley and Tollervey, 2009; Moore and Proudfoot, 2009). How these different steps are carried out and what proteins are involved in these processes has been the focus of gene expression studies over the last few decades. The ability to detect and quantify mRNA levels was thus the key requirement. Traditionally, mRNA detection is achieved using some kind of hybridization technique. While Northern blots are able to detect only a few mRNAs at the time, array technologies now allow expression studies of an entire organism in a single experiment (Ausubel, 1988; Coppée, 2008; Holstege *et al.*, 1998).

One limitation of arrays or Northern blots, however, is that large numbers of cells are required to isolate sufficient material to perform an experiment. Additionally, cells must be broken up to isolate RNA and RNA get lost or degraded during the isolation procedure. Therefore, spatial information gets lost. The steps along the gene expression pathway occur in different cellular compartment and preserving spatial information is often critical to understand cellular processes. Furthermore, variability among different cells in a population cannot be observed by ensemble measurements. Cells from different cell cycle or developmental stages express unique sets of genes and such alternate expression profiles are obscured when pooling cells. Finally, expression "noise" resulting from stochastic fluctuations in biological processes cannot be observed without single cell analysis (Elowitz *et al.*, 2002; Kaufmann and van Oudenaarden, 2007).

These limitations are circumvented by single cell analysis (Kaufmann and van Oudenaarden, 2007; Zenklusen *et al.*, 2008). Spatial information and cell-to-cell differences become easily observed when molecules are detected in single cells, made possible by the extensive use of fluorescent

proteins (Shaner *et al.*, 2007). To analyze mRNA expression, however, single cell techniques are less widely used. Fluorescent *in situ* hybridization (FISH) is the most robust and straight-forward method for single cell mRNA analysis (Dong *et al.*, 2007; Long *et al.*, 1997; Zenklusen *et al.*, 2008). To detect mRNA in cells, fluorescently labeled probes are hybridized to fixed cells immobilized on glass slides. The technique is noninvasive, as no genetic modifications are necessary. Choosing well-designed probes coupled with bright fluorescent dyes allows the detection of single mRNA molecules in single yeast cells (Dong *et al.*, 2007; Femino *et al.*, 1998; Long *et al.*, 1997; Zenklusen *et al.*, 2008). The applications of this technique in gene expression analysis have a wide range; we have used FISH to study transcription, splicing, and mRNA localization (Dong *et al.*, 2007; Long *et al.*, 1997; Zenklusen *et al.*, 2008).

Yeast is an ideal system to perform single molecule expression analyses. Many genes in yeast are expressed at a very low level of less than 10 copies per cell (Holstege *et al.*, 1998; Zenklusen *et al.*, 2008). Therefore, ''absolute'' quantification of mRNA expression can be performed; the number of mRNA molecules can be determined simply by counting. The small size of a yeast cell is also advantageous in this case, allowing analysis of expression levels in many single cells simultaneously. Expression and localization studies can, therefore, be performed with unprecedented precision.

In this chapter, we will progress through the different steps of performing a single mRNA resolution FISH experiment. We begin with how probes are designed and labeled before we describe a step-by-step protocol for FISH. Finally, we briefly describe some aspects of data analysis.

## 2. Probe Design

A crucial step of a successful FISH experiment is designing FISH probes. To achieve single molecule sensitivity, multiple oligonucleotide probes, each labeled with up to five fluorescent dyes are hybridized to an mRNA (Fig. 26.1). To allow the coupling of multiple dyes onto one probe, a minimal probe length is required. Probes should also be long enough to ensure high specificity and allow stringent hybridization conditions. Probes of around 50 nucleotides (nt) in length with about 50% CG content typically work best, demonstrating high specificity under stringent hybridization conditions. As multiple probes against one gene are used, it is important to design probes with similar melting temperature. Using these standard settings (50 nt/50% CG) during probe design also facilitates the simultaneous use of differentially labeled probes against multiple target mRNAs (Fig. 26.1).

Probes are designed using commercial DNA sequence analysis software such as Oligo (Molecular Biology Insights, Inc.). To find target sites,

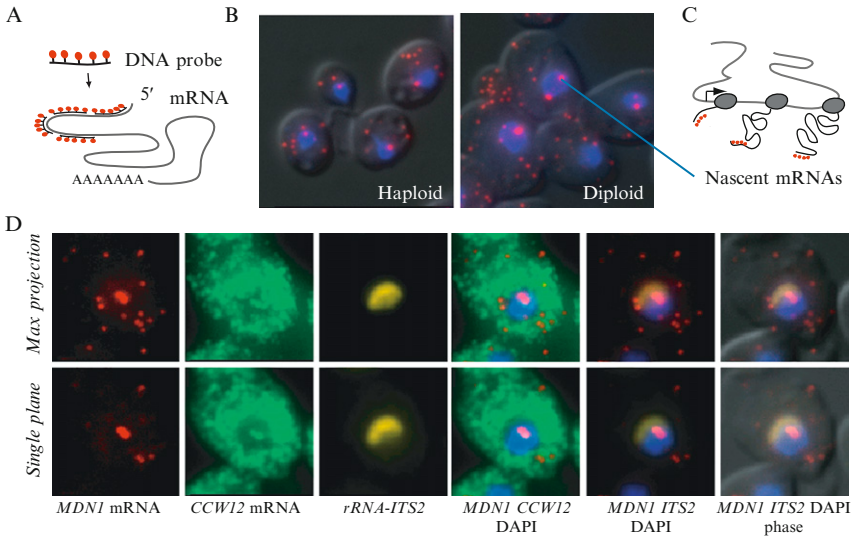



**Figure 26.1** Single mRNA sensitivity fluorescent *in situ* hybridization (FISH). (A) Schematic diagram of the FISH protocol. A mix of four 50 nt DNA oligonucleotides, each labeled with five fluorescent dyes, is hybridized to paraformaldehyde fixed yeast cells to obtain single transcript resolution. (B) Single mRNA FISH for *MDN1* mRNA. Single mRNAs are detected in the cytoplasm, higher intensity spot in the nucleus. Haploid and diploid yeast cells are shown. Probes hybridize to the 5′ of the mRNA. *MDN1* mRNA (red), DAPI (blue), and DIC. (C) Cartoon illustrating that the number of nascent mRNAs at the site of transcription is used to determine the polymerase loading on a gene using probes to the 5′end of *MDN1*. (D) Nascent transcripts of neighboring genes colocalize at the site of transcription. Diploid cells are hybridized with probes against *MDN1* (red) and *CCW12* (green). Nucleolus is stained with probes against the ITS2 spacer of the rRNA precursor (yellow). Maximum projection of 3D-dataset and single plane containing the transcription sites are shown (Zenklusen *et al.*, 2008).

the gene of interest is scanned for 50 nt complementary sequences with ~50% CG content. If none fitting the criteria can be found, the length of the probe can be adjusted by adding or removing a few bases while keeping a similar melting temperature. It is important not use probes forming stable secondary structures as this may interfere with efficient hybridization. Avoid using probes forming internal stem loops with a $\Delta G > -2.5$ kcal/mol. Probes should also be tested for cross-hybridization to other genes, for example, by using Blast in SGD™ (Saccharomyces Genome database). Strong sequence homology is rare but can challenge probe design, for example, when designing probes for ribosomal protein genes, present in two copies per genome with strong sequence homology.

To incorporate multiple labels into a single DNA oligonucleotide probe, modified bases are inserted during synthesis. Inserting amino-allyl dTs

allows efficient coupling with most commercially available dyes after synthesis. To avoid quenching of dyes, modified bases should be spaced by 8–10 nt. Different companies synthesize oligos containing internal labels, but due to relatively high costs it is often preferable to synthesize the probes on site if a DNA synthesis facility or a DNA synthesizer is available. Alternatively, probes containing a single modified base can be used. Such probes are synthesized by most companies and are much cheaper compared to probes bearing multiple labels. However, more probes have to be used to allow single molecule detection (Raj *et al.*, 2008).

## 3. Probe Labeling

Single molecule detection requires high-labeling efficiencies. We use cyanine dyes, containing a monofunctional NHS-ester for efficient coupling to amino-allyl Ts. Cy3, Cy3.5, and Cy5 (CyDye™, GE Healthcare) work well, but other dyes with monofunctional NHS-ester from other companies can be used. Dyes in the green (emission below 500 nm) are less well suited for FISH in yeast as cells show more background fluorescence and single molecule detection becomes difficult.

Labeling is done as described by the manufacturer with minor modifications. We prepurify probes prior to labeling using a QIAquick Nucleotide Removal Column (Quiagen), as this has been shown to increase labeling efficiency. Five micrograms of DNA oligonuleotide is labeled using a single Amersham Cy3, Cy3.5, or Cy5 dye pack. When multiple probes against one gene are used, probes can be pooled in equal molar ratios and the probe mix is labeled together.

Labeling efficiency is determined by measuring absorption in a spectrophotometer. If available, use a NanoDrop (Thermo Fisher), which allows measuring of low volumes (1 $\mu$l), therefore, reducing probe loss. Labeling efficiency is calculated using a formula that corrects for absorption of the fluorophore at 260 nm. Labeling of $>90\%$ should be obtained. For unknown reasons, labeling efficiency of Cy3.5 is generally lower (75–80%).

### 3.1. Materials

- DNA oligonucleotide containing amino-allyl modified Ts
- Mono-Reactive CyDye™ Cy3, Cy3.5, and Cy5 (GE Healthcare, #PA23001, PA23501, PA25001)
- QIAquick Nucleotide Removal Kit (Qiagen #28304)
- Spectrophotometer
- Labeling buffer (0.1 *M* sodium bicarbonate, pH 9.0)

## 3.2. Protocol

1. Measure concentrations of unlabeled DNA oligonucleotides.
2. When using multiple probes against one gene, combine probes to total of 5 $\mu$g of probes per labeling. For example, when using four probes to gene A, use 1.25 $\mu$g of each).
3. Add 500 $\mu$l of buffer PN from QIAquick Kit, mix.
4. Purify on QIAquick column according to the protocol. To increase binding, load the sample twice onto the same column.
5. Elute probes from columns using 40 $\mu$l $H_2O$. Do not use the elution buffer from the kit.
6. Lyophilize probes in a SpeedVac.
7. Resuspend the DNA pellet in 10 $\mu$l labeling buffer and add to the dye containing tube.
8. Resuspend the dye by vortexing vigorously and then perform a quick spin to collect the labeling reaction at the bottom of the tube.
9. Incubate in the dark at room temperature overnight.

*Purify the probes from the free dye using QIAquick columns:*

10. Add 500 $\mu$l of buffer PN to the labeling reaction and load onto column.
11. Spin through columns according to the protocol.
12. Load the flow-through a second time onto the same column to increase probe recovery.
13. Spin through columns according to the protocol.
14. Wash column twice with buffer PE to remove all nonincorporated dye.
15. Elute the labeled probes using 100 $\mu$l of elution buffer.
16. Measure concentration and labeling efficiency using a spectrophotometer.
17. Store probes at $-20$ °C in the dark.

## 3.3. Measuring labeling efficiency

To calculate the labeling efficiency, the extinction coefficient and the absorbance of the dye and the oligo at 260 nm and the emission peak of the dye have to be considered. The molar extinction coefficient ($\varepsilon$) of the DNA oligonucleotides is calculated as described by Beer–Lambert law (Cavaluzzi and Borer, 2004). A web site from an oligo synthesis company could be used for the calculation (we use http://www.idtdna.com/analyzer/Applications/OligoAnalyzer). To calculate the molecular weight of the amino-modified oligo, add 179.16 g/mol per modified base to the calculated molecular weight of the unmodified oligo.

The exact DNA concentration [DNA] is calculated using Eq. (26.1), the dye concentration [Dye] using formula (26.2). The labeling efficiency is then determined by dividing the [Dye]/[DNA] by the number of modified

bases on the probe (26.3). $A_{DNA}$ is the absorption of the sample at 260 nm. $A_{dye}$ is the absorption at absorbance max of the dye. $\varepsilon_{dye}$ is extinction coefficient of the dye, $\varepsilon_{DNA}$ the extinction coefficient of the DNA:

$$[\text{DNA}] = \frac{A_{\text{DNA}} - \varepsilon_{\text{dye}(260)} \times (A_{\text{dye}}/\varepsilon_{\text{dye}(\max)})}{\varepsilon_{\text{DNA}} \times 0.1\,\text{cm}} \tag{26.1}$$

$$[\text{Dye}] = \frac{A_{\text{dye}(\max)}}{\varepsilon_{\text{dye}} \times 0.1\,\text{cm}} \tag{26.2}$$

$$\text{Labeling efficiency} = \frac{[\text{Dye}]}{[\text{DNA}]} \times \frac{1}{5} \tag{26.3}$$

Extinction coefficients of the dyes at 260 nm ($\varepsilon_{260}$) and their absorption maximum ($\varepsilon_{max}$) are shown in the table as follows:

| Dye | $\varepsilon_{260}$ | $\varepsilon_{max}$ | Absorbance (nm) | Emission (nm) |
|---|---|---|---|---|
| Cy3 | 12,000 (8%) | 150,000 | 550 | 570 |
| Cy3.5 | 40,800 (24%) | 170,000 | 581 | 596 |
| Cy5 | 12,500 (5%) | 250,000 | 649 | 670 |

## 4. Cell Fixation, Preparation, and Storage

To prepare cells for FISH, cells are grown in the appropriate media and fixed by adding paraformaldehyde directly to the media. After extensive washes, the cell wall is removed using lyticase. Cells are digested in an isotonic buffer to prevent cells from bursting after the cell wall has been removed. Cells also become very fragile and strong shearing forces (extensive pipetting and vortexing) will break the cells open, so gentle handling is required. Complete digestion, however, is necessary to obtain optimal FISH results. Progression of the digest is, therefore, observed by visual inspection using phase contrast. Cells will turn dark when the cell wall is digested away, whereas undigested cells look transparent. Avoid digesting cells for too long, as overdigestion can lead to cell lysis.

Following digestion, cells are attached to coverslips. Using round 18 mm cover glass slips allows most subsequent steps to be performed in 12-well tissue culture plates. The cover glass is coated with poly-L-lysine for cells to attach. Alternatively, precoated coverslips can be purchased from different vendors. Cells are spotted on coverslips and allowed to settle by gravity. Unadhered cells are washed off and coverslips are finally stored in 70%

ethanol at −20 °C. Ethanol dissolves membranes allowing better penetration of probes during the hybridization step and serves at the same time as a preservative, permitting cells to be stored for many months.

## 4.1. Materials

- Paraformaldehyde 32% solution, EM grade (Electron Microscopy Science #15714)
- Lyticase (Sigma # L2524, resuspend in 1× PBS to 25,000 U/ml. Stored at −20 °C)
- Ribonucleoside–vanadyl complex (VRC; NEB #S1402S)
- β-Mercaptoethanol
- Sorbitol
- 1 *M* $KHPO_4$, pH 7.5
- 70% ethanol
- Noncoated coverslips (Fisherbrand Cover Glasses Circles No. 1: 0.13–0.17 mm thick; size: 18 mm (#12-545-100)) or
- Precoated coverslips (Fisherbrand Coverglass for growth 18 mm (12-545-84))
- Poly-L-lysine (#P8920)
- 12-well cell culture plates

*Solutions to be prepared:*

| | |
|---|---|
| • Buffer B | 1.2 *M* sorbitol, 100 m*M* $KHPO_4$, pH 7.5 |
| • Spheroplast buffer | 1.2 *M* sorbitol<br>100 m*M* $KHPO_4$, pH 7.5<br>20 m*M* ribonucleoside–vanadyl complex (VRC; NEB #S1402S)<br>20 m*M* β-mercaptoethanol<br>Lyticase (25 U lyticase per OD of cells) |
| • Respuspention buffer | 1.2 *M* sorbitol<br>100 m*M* $KHPO_4$, pH 7.5<br>20 m*M* ribonucleoside–vanadyl complex |

## 4.2. Protocol

- *Growth and fixation*
  1. Grow 50 ml BY4741 cells in YPD in a 250-ml flask at 30 °C on an orbital shaker to an optical density at 600 nm (OD 600) of 0.6.
  2. Prepare a 50-ml Falcon tube containing 6.3 ml of 32% (v/v) paraformaldehyde. Paraformaldehyde is toxic, wear gloves and handle in the fume hood!

3. Fix cells by transferring 43.7 ml of culture to a 50-ml tube containing the paraformaldehyde (final concentration of 4%, v/v) and mix.
4. Incubate cells for 45 min at room temperature on a tabletop shaker.
5. Collect cells by centrifugation using a swinging bucket rotor at 3500 rpm at 4 °C.
6. Wash cells three times with 10 ml of cold buffer B.
7. Resuspend cells in 1 ml buffer B and transfer cells to a 1.5-ml Eppendorf tube.
8. Pellet cells using tabletop centrifuge (3 min, 4000 rpm).

- *Digestion*

9. Resuspend cells in 1 ml spheroplast buffer plus 30 $\mu$l of lyticase (at 25 U/$\mu$l).
10. Incubate cells at 30 °C for 8 min.
11. Check the progression of the digest using a phase contrast microscope. Place 3.5 $\mu$l on a microscope slide, cover with a coverglass and inspect digestion using a 20× objective. Undigested cells are transparent while digested cells will turn dark. If >80% of cells are digested proceed to step 12. If fewer cells are digested, continue incubation and check for digestion every 2–3 min.
12. Collect cells by centrifugation for 3 min at 3500 rpm at 4 °C. Do not spin at a higher speed or cells will break.
13. Wash cells with 1 ml of cold buffer B (pipette carefully).
14. Resuspend pellet in 1.5 ml of buffer B, keep on ice.

- *Attaching cells to coverslips*

15. Place poly-L-lysine treated 18 mm round coverslips face up into 12-well tissue culture dishes, one coverslip per well.
16. Drop 150 $\mu$l of cells to the center of a coated coverslip.
17. Let cells settle for 30 min at 4 °C.
18. Slowly add 2 ml of buffer B to each well, then remove buffer B using a vacuum aspirator. This will remove cells not attached to the coverslip and leave a monolayer of immobilized cells.
19. Slowly add 2 ml of 70% ethanol of each well.
20. Store cells for at least 3 h at −20 °C. Cells can be stored at −20 °C for at least 6 months.

- *Prepare poly-L-lysing coverslips*

Carefully put one box of 18 mm round coverslips into 500 ml 0.1 N HCl and boil for 10 min. Rinse extensively with $H_2O$, autoclave and store in 70% ethanol.

To coat coverslips with poly-L-lysine, place 100 $\mu$l of a 0.01% (w/v) poly-L-lysine solution onto a coverslip, incubate at room temperature for 5 min, remove the solution using a vacuum pump and let the remaining liquid dry. Then wash twice with $H_2O$ and let air dry. The poly-L-lysine coated coverslips can be stored for several months.

## 5. Hybridization

Only very low probe concentrations are needed in the hybridization reaction to allow single mRNA detection. Generally, 0.5 ng per probe per hybridization reaction is sufficient. To block nonspecific binding of the probes, competitor DNA and RNA is added in large excess to the hybridization solution.

The formamide concentration in the hybridization mix and the subsequent wash steps is critical to get optimal hybridization specificity. Generally, we use 40% formamide for standard probes (50 nt/50% CG), but if high background is observed, increasing the formamide concentration from 40% to 50% can reduce background. To detect the entire pool of polyA, mRNAs in the cell can be detected using a 50-nt poly-dT probe, but the formamide concentration has to be reduced to 15%.

For the hybridization step, the coverslip with the immobilized cells are inverted onto a droplet of the hybridization solution. Floating of the coverslip on the hybridization solution leads to even distribution of hybridization solution and the best results. This works much better than using multiwell microscope slides. Hybridization is done in hybridization chamber overnight at 37 °C. The chamber is a simple, self-assembled unit consisting of a glass plate and two Parafilm layers separated by cardboard spacers (Fig. 26.2).

After hybridization, the coverslips are placed back into a 12-well plates and washed extensively to ensure that all unbound probes are removed. After a short wash in a DAPI containing solution, cells are mounted and are ready to be imaged.

### 5.1. Materials

- Glass plate, about 20 × 20 cm
- Parafilm
- Cardboard spacers
- 12-well cell culture plates
- Glass slide

*Solutions to be prepared:*

- 40% formamide/2× SSC
- 2× SSC/0.1% Triton X-100
- 1× SSC
- 1× PBS
- Solution F (40% formamide, 2× SSC, 10 m*M* $NaHPO_4$, pH 7.5)
- Solution H (2× SSC, 2 mg/ml BSA, 10 m*M* VRC)

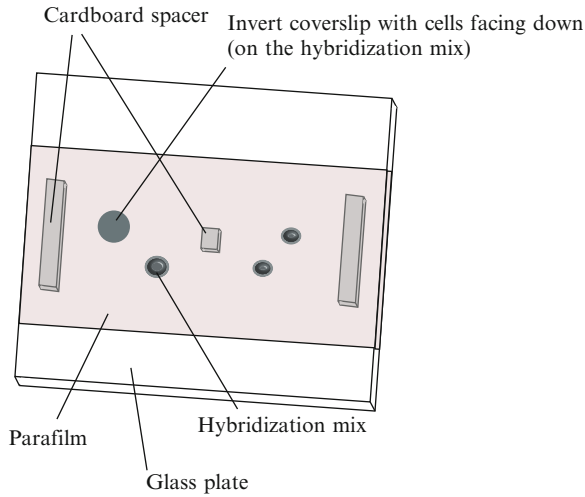



**Figure 26.2** **Hybridization chamber. The hybridization chamber is assembled using a glass plate, Parafilm and cardboard spacers. The coverslips with cells are inverted onto a drop of the hybridization solution placed onto the first Parafirm layer. To seal the chamber, a second layer of Parafilm is placed on top of the coverslips. To keep the second Parafilm layer from touching the coverslips, cardboard spacers are placed on both sides and in the middle of the first Parafilm layer. The interior volume of the chamber is small and evaporation is not a problem at 37 °C. However, the two layers of Parafilm have to be properly sealed to prevent evaporation.**

- *Escherichia coli* tRNA (Roche # 10 109 541 001)
- ssDNA (deoxyribonucleic acid, single stranded from salmon testes, Sigma #D9156)
- DAPI solution (0.5 $\mu$g/ml DAPI (Sigma #D9564) in 1× PBS. Store at 4 °C in the dark)
- Mounting solution (ProLong® Gold antifade reagent (Invitrogen # P36934))

## 5.2. Probes used for the hybridization shown in Figs. 26.1 and 26.3

Bold Ts represent amino modified bases

| | |
|---|---|
| *MDN1* probes (Cy3) | |
| MDN1-794 | TT**T** GTC GTG GAT AG**T** GTG GAC CT**T** AGG GAC GA**T** AAC GCC ACA GAT **T**GA CG |
| MDN1-860 | C**T**C CCG AGT **T**GA CGA AGA GAG GAA ACC G**T**T TTA TGA G**T**A GGG ACA AAG G**T**T |

(*continued*)

(*continued*)

| | |
|---|---|
| MDN1-1104 | C**T**A TAA GTA CCC A**T**C TCC CTT CTT **T**GA CCG CGG **T**AG CGA GAA CAC CAG C**T**C |
| MDN1-1210 | TT**T** GCA GCC TTT ACA GTC **T**CT CCT CTG GA**T** GGA ATG GT**T** AGT TCG CGC **T**T |
| *CCW12* probes (Cy3.5) | |
| CCW12-59 | GG**T** GAC CAA AGT GG**T** AGA TTC TTG GC**T** GAC AGT AGC AG**T** GGT AAC GT**T** AG |
| CCW12-140 | G**T**C ATC GAC GG**T** GAC GGT AGC GG**T** GGA AAC CAA AGC **T**GG GGA GAC AGT **T**T |
| CCW12-191 | C**T**T TGG GGC T**T**C AGT GGT CAA TGG GCA CCA GG**T** GGT GTA TTG AGT GA**T** AA |
| CCW12-245 | GG**T** GTT CTT TGG AGC **T**TC AGT AGA GG**T** AAC TGG AGC AGC AG**T** AGA AGT AC |
| *rRNA-ITS2* (Cy5) | |
| ITS2-1 | A**T**A GGC CAG CAA TTT CAA GT**T** AAC TCC AAA GAG TAT CAC **T**C |

## 5.3. Protocol

1. Remove the ethanol from the 12-well plate using a vacuum pump and rehydrate samples by adding 2 ml 2× SSC at RT for 5 min. Do this twice.
2. Wash cells once with 40% formamide/2× SSC at RT for 5 min.

*During washes, prepare the hybridization mix:*

3. Mix 0.5 ng of each probe per coverslip with 10 μg of *E. coli* tRNA and 10 μg of ssDNA (2 ng of probe mix when using four probes against one gene).
4. Lyophilize using a SpeedVac.
5. Add 12 μl of solution F to probe tube, heat at 95 °C for 3 min.
6. Add 12 μl of solution H to the hybridization mix.
7. Put a drop of 22 μl of hybridization mix onto the Parafilm stretched out on a glass plate. Avoid bubbles in the hybridization mix. (Use the back of a forceps to scratch the edges of the Parafilm so that the Parafilm sticks to the glass plate.)
8. Using forceps, place the coverslip with cells facing down on the hybridization mix. No bubbles should form. Multiple coverslips can be placed next to each other onto a single glass plate, but leave about 1.5 cm space between coverslips.
9. To seal the ''hybridization chamber,'' place two cardboard spacers (2–3 mm thick and 5 × 0.5 cm in length) on opposite sides of the

glass plate over the Parafilm plus a 0.5 × 0.5 cm place onto the centre of the plate. Cover the glass plate with a second layer of Parafilm, without touching the coverslips. Seal the two layers of Parafilm using the back of the forceps to avoid evaporation. Cover with aluminum foil.

10. Incubate at 37 °C over night in the dark.
11. Preheat 40% formamide/2× SSC at 37 °C, put 2 ml in 12-well tissue culture dish.
12. Place cover slips back in 12-well tissue culture dish containing 40% formamide/2× SSC, cells facing up; incubate 15 min at 37 °C (incubator).
13. Wash once more with 40% formamide/2× SSC at 37 °C (2 ml, 15 min).
14. Wash once with 2× SSC 0.1% Triton X-100 at RT (2 ml, 15 min).
15. Wash once with 1× SSC at RT (2 ml, 15 min).
16. Wash coverslip in 1× PBS plus DAPI (2 ml, 2 min).
17. Wash 1× with 1 × PBS (2 ml, 2 min).
18. Before mounting, dip coverslip in 100% EtOH, let them dry.
19. Invert cells facing down onto a drop of mounting solution placed on a glass slide. Allow the mounting solution to polymerize over night at room temperature in the dark.
20. Seal coverslips with nail polish. Let nail polish dry before imaging, otherwise the objective may be damaged.
21. Go to the microscope and enjoy your images.

Slides can be stored at 4 °C for a few days and at −20 °C for months in the dark.

## 6. Image Acquisition

The need for sensitive imaging equipment was likely one reason why single molecule detection was not approachable in the past. However, since sensitive CCD cameras have become a standard component of most microscopes and dyes are very bright and photostable, signal intensities are not a limiting factor for detection of single mRNAs by FISH. Most epifluorescence microscope setups in imaging facilities are sensitive enough to detect single mRNAs. We use a standard epifluorescent microscope and CCD camera (described below).

When simultaneously imaging mRNAs expressed from multiple genes using probes labeled with different fluorophores, it is crucial to use the correct filter sets to avoid bleedthrough between the different channels. For example, when using Cy3 and Cy3.5, whose absorbance and emission are relatively close to each other (550/570 nm and 581/596 nm) narrow band pass filter sets have to be used. Appropriate filter sets are listed below.

To obtain expression profiles and mRNA distributions, images have to be acquired in 3D. Using a 100× objective, collect z-slices every 200 nm. Using the setup presented below, exposure times of 1 s per z-stack should lead to sufficient signal. If single mRNAs cannot be detected, it is likely that the hybridization did not work or the microscope is not aligned properly.

### 6.1. Microscope (example)

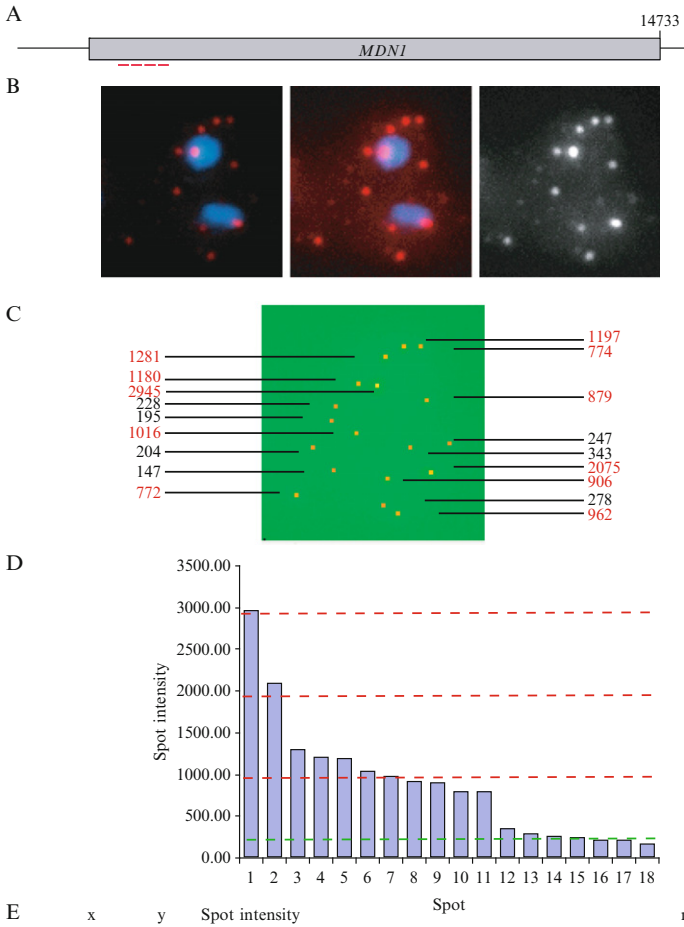
- Olympus BX61 epifluorescence microscope (Olympus, Center Valley, PA)
- Olympus UPlanApo 100×, 1.35 NA oil-immersion objective
- Olympus U-DICTHC Nomarski prism for DIC
- Chroma Filters 31000 (DAPI), 41001 (FITC), SP-102v1 (Cy3), SP-103v1 (Cy3.5), and CP-104 (Cy5) (Chroma Technology, Rockingham, VT)
- Light source X-Cite 120 PC (EXFO, Mississauga, ON)
- CoolSNAP HQ camera (Photometrics, Tucson, AZ)

## 7. Image Analysis

Hybridizing four to five FISH probes, each labeled with five fluorescent dyes to an mRNA creates a strong fluorescent signal. Although barely visible by eye, single mRNAs are easily detected using a standard CCD camera. Single mRNAs signals appear as diffraction limited spots within the cell. Sites of transcription often show higher signal intensities and are easily distinguishable as they colocalize with the DAPI signal (Figs. 26.1 and 26.3). *MDN1* transcription sites are visible by eye and being able to see a *MDN1* transcription site by eye is a good first indicator for a successful FISH experiment.

To simplify the data analysis, it is often helpful to reduce the 3D dataset to a 2D image using a maximum projection. The maximum projection displays the maximum value of all images in the z-stack for particular pixel locations and creates a 2D image. As mRNAs for most genes are expressed at low numbers, the probability that two mRNAs are found in the same $x$–$y$ but a different $z$ position is low, allowing a reduction to 2D to accurately represent the 3D dataset.

To test for specificity of the signal, probes can be hybridized to control cells not expressing the transcript of interest, for example, a deletion strain. Alternatively, a gene can be put under an inducible promoter, like a GAL promoter and transcription turned off long enough that all mRNAs are degraded. Using well-labeled probes and high hybridization efficiency, the difference in signal between cells expressing and not expressing is generally




E

| x | y | Spot intensity | | | mRNAs per spot |
|---|---|---|---|---|---|
| 55.4 | 74.3 | 2945.7 | | | 3.0 |
| 80.7 | 31.7 | 2075.4 | | Intensity of sites of transcription | 2.1 |
| 59.2 | 87.7 | 1281.6 | | | 1.3 |
| 68.4 | 92.5 | 1197.9 | | | 1.2 |
| 46.1 | 74.6 | 1180.1 | | | 1.2 |
| 44.7 | 50.8 | 1016.4 | | | 1.0 |
| 65.0 | 12.3 | 962.8 | | | 1.0 |
| 60.2 | 28.9 | 907.0 | | | 0.9 |
| 78.7 | 66.7 | 879.8 | | | 0.9 |
| 76.2 | 93.1 | 774.3 | | | 0.8 |
| 16.1 | 21.4 | 772.9 | 996.9752 | Average intensity of a single mRNA | 0.8 |
| 71.2 | 44.3 | 343.7 | | | 0.3 |
| 58.5 | 15.9 | 278.2 | | | 0.3 |
| 90.4 | 46.2 | 247.6 | | | 0.2 |
| 35.3 | 64.3 | 228.3 | | | 0.2 |
| 23.8 | 44.3 | 204.8 | | | 0.2 |
| 32.6 | 56.8 | 195.2 | | | 0.2 |
| 34.1 | 32.7 | 147.4 | 235.0319 | Average intensity of a single probe | 0.1 |

**Figure 26.3** Quantifying mRNA signal intensities. Intensity of a single mRNA can be calculated by determining the fluorescence intensity emitted from a single probe. (A) FISH probes hybridizing to the 5′ end of *MDN1* mRNA were used for hybridization shown in (B). A small number of single probes tend to hybridize unspecifically to the cells and can be visualized by changing the contrast levels (B, compare left and

obvious. Spots are observed throughout the cell when a gene is expressed and no signal should be observed in cells where the gene is not expressed.

However, single molecule resolution FISH is not completely devoid of background (Fig. 26.3). When analyzing the images carefully, low-intensity signals are found in the negative control. The weak signals originate from single FISH probes sticking nonspecifically to the cell. Despite sequence specificity and stringent hybridization and washing conditions, a low number of single probes will usually stick to the cell. Their signal intensity is low, and they appear as weaker diffraction limited spots compared to the signal emitted from an mRNA. These signals can be distinguished from an mRNA signal. In most cases, the difference is obvious, mRNA signals are bright and single probe signals are low. However, sometimes this difference is not so evident. Distinguishing between background-sticking and real mRNA signal particularly becomes an issue if hybridization efficiency of the probes is low. In this case, some mRNAs will only have one, two, or three out of four possible probes bound, resulting in signal with variable intensities for different mRNAs within a single cell. When two or less probes are bound, the signal becomes more difficult to separate from a single probe nonspecifically bound to the cell.

There are two ways to determine the signal intensity of a single probe and to distinguish them from mRNA signals. The first uses a rough approximation of the signal intensities of single probes. Similar to nonspecifically sticking to cells, a small number of probes will also stick to the glass surface outside of the cells. When using well-labeled probes, their signal intensity is homogenous and they are easily distinguishable from other ''junk'' on the glass. Use image acquisition software to determine the brightest pixel of each spot. Signal intensities as low as signals from spots on the glass slide indicate background, while higher intensity signals originate from mRNA signals. However, it is important to notice that using this method, the autofluorescence from the cell, although usually low, is added to the signal emitted from a single probe within a cell but not the one from the glass. Therefore, using the intensity of a single probe from the glass background will underestimate the signals expected from mRNAs inside the cell. This method is simple, although only approximative in distinguishing background spots from real signals.

A better and more quantitative approach is to determine the exact signal intensity emitted from each mRNA. Different spot detection and

**middle panel). The intensity is determined using a spot detection program. (C) Signal intensity of each spot corresponding to a single DNA probe is shown in black, signal intensities of single mRNA and sites of transcription are shown in red. Consistent with the four probes used in the hybridization (A), intensity of single mRNA signals in the cytoplasm is four times the intensity of a single probe (D, E). Nascent mRNAs at the site of transcription are two and three times the intensity of a single mRNA in the cytoplasm (Zenklusen *et al.*, 2008).**

quantification algorithms exist and one of the most established methods determines the signal intensity emitted from a diffraction limited spot by fitting a 2D Gaussian mask over each spot (Thompson *et al.*, 2002). We have developed custom software to apply this algorithm, which also takes into account a background correction and can be found at http://www.singerlab.org (Zenklusen *et al.*, 2008). Shown in Fig. 26.3 are two cells hybridized with four probes to the 5′ of the *MDN1* mRNA. The spot detection program identifies 18 spots. Spots containing a single or four probes can easily be distinguished from each other. Single probes intensities are around 230 a.u. and mRNA signals show a mean intensity of 996 a.u., four times the intensity of a single probe. This illustrates how signals of nonspecific probe binding can be distinguished from signals of probes hybridized to mRNA molecules. Determining the intensity of single probes also allows to establish the signal intensity that is expected from an efficient hybridization and thereby allows to determine hybridization efficiency.

Figure 26.3 furthermore illustrates why achieving high hybridization efficiency is crucial. Low hybridization efficiency will lead to datasets that are difficult to analyze, as a clear distinction between signal and background is not possible. When signal intensity of individual mRNAs is highly heterogeneous, it is best to repeat the hybridization to obtain more uniform signals. For some probe sets, efficient hybridization can not be achieved and new probes against different regions in a gene will have to be synthesized.

The ability to determine the intensity of a single mRNA also allows calculation of the number of nascent mRNAs at the site of transcription. Dividing the signal intensity of the two spots colocalizing with the DAPI signal in Fig. 26.3 shows that two respectively three nascent mRNAs are present on the *MDN1* genes. Determining the number of nascent transcripts is a measure of polymerase loading and therefore the most direct assessment for transcriptional activity on a single gene. Importantly, to determine polymerase density on a gene, probes hybridizing to the 5′ end of the mRNAs have to be used.

Quantification of signals from highly expressed genes is more difficult. As shown in Fig. 26.1, *CCW12* is highly expressed and individual mRNAs overlap each other so that it is not possible to determine the intensity of every single mRNA. Therefore, this technique is better suited to study genes expressed at low levels.

## 8. Summary and Perspectives

Single molecule resolution FISH is a powerful tool to study gene expression. We have applied it to count single mRNAs and determine transcription kinetics, investigate splicing regulation, and study mRNA

localization. However, its potential applications are even broader. There are many aspects of gene expression regulation where using single molecule resolution FISH will be a useful tool because it is able to detect and count every individual mRNA molecule in a cell. Even if expressed at only one molecule per cell, mRNAs can be detected and the precise location within the cell can be determined. Studies of transcription networks as well as more classical gene expression processes like mRNA export and degradation can be analyzed with greater detail using single molecule methodologies. The ability to detect single molecules will expand our understanding of these cellular processes.

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