

Single-probe RNA FISH in Yeast

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[Abstract] Quantitative profiling of mRNA expression is an important part of understanding the state of a cell. The technique of RNA Fluorescence *In Situ* Hybridization (FISH) involves targeting an RNA transcript with a set of 40 complementary fluorescently labeled DNA oligonucleotide probes. However, there are many circumstances such as transcripts shorter than 200 nt, splicing variations, or alternate initiation sites that create transcripts that would be indistinguishable to a set of multiple probes. To this end we adapted the standard FISH protocol to allow the use of a single probe with a single fluorophore to quantify the amount of transcripts inside budding yeast cells. In addition to allowing the quantification of short transcripts or short features of transcripts, this technique reduces the cost of performing FISH.

Keywords: RNA FISH, Fluorescence *In Situ* Hybridization, *Saccharomyces cerevisiae*, Budding yeast, Transcription, Single molecule

[Background] Precise quantification of the transcript profile of single cells is possible by single molecule Fluorescence *In Situ* Hybridization (smFISH). This procedure gives good signal to noise by targeting a single mRNA molecule with multiple fluorescently labeled DNA oligo probes (Raj and Tyagi, 2010). Using this scheme, mRNA of length shorter than 200 nucleotides cannot be detected. However, in most experiments, the absolute transcript copy number is less informative than the relative copy number. To detect short transcripts or sequences, a short single DNA oligo probe can be used. The detection efficiency of a single probe is greater than 50 percent when using a single fluorophore to count mRNA (Wadsworth *et al.*, 2017).

Materials and Reagents

1. Pyrex bottle (Corning, PYREX™, catalog number: 13951L)
2. Falcon tube 50 ml (VWR, catalog number: 89039-658)
3. Falcon tube 15 ml (VWR, catalog number: 89039-666)
4. Nitrile gloves (VWR, catalog number: 40101-348)
5. Light-duty tissue wipers (VWR, catalog number: 82003-820)
6. Lens cleaning tissues (Olympus, catalog number: C-0100)
7. Aluminum foil
8. Pipette tips (VWR, catalog numbers: 89079-466, 89079-460, and 89079-472)
9. Plastic cuvettes (BrandTech Scientific, catalog number: 759075D)
10. Culture flask (Corning, PYREX™, catalog number: 4442-250)

11. Microcentrifuge tube (Corning, Axygen®, catalog number: MCT-175-C)
12. Microcentrifuge tube rack (Thermo Fisher Scientific, catalog number: 5973-0015)
13. Petri dish (VWR, catalog number: 25384-088)
14. #1.5, 18 mm square coverslip (Fisher Scientific, catalog number: 12-518-108B)
15. Glass slide (Fisher Scientific, catalog number: 12-544-1)
16. *Saccharomyces cerevisiae* strains (collaborators or ATCC)
17. Low Auto Fluorescence Immersion Oil (Thorlabs, catalog number: MOIL-30)
18. Ethanol (VWR, catalog number: BDH1156)
19. Methanol ≥ 99% ACS Spectrophotometric grade (Sigma-Aldrich, catalog number: 154903-2L)
20. RNase free water (Quality Biological, catalog number: 351-068-131)
21. Fluorophore labeled DNA oligo probes, HPLC purified (Integrated DNA technologies or Eurofins Scientific)
22. High Strength 5-min Epoxy (Amazon B001QFGTHG)
23. Zymolyase-20T at 21,000 units/g (Zymolyase-20T, Seikagaku Business Corporation)
24. SD Complete (see Recipes)
 - a. Carbon, Nitrogen, and Salts (CNS)
 - Dextrose (Sigma-Aldrich, catalog number: G8270-25KG)
 - Ammonium sulfate (Sigma-Aldrich, catalog number: A4418-5KG)
 - Potassium phosphate monobasic (VWR, catalog number: MK710002)
 - Magnesium sulfate (Sigma-Aldrich, catalog number: M2773-500G)
 - Sodium chloride (Fisher Scientific, catalog number: S671-500)
 - Calcium chloride (Sigma-Aldrich, catalog number: C3306-250G)
 - Biotin (Sigma-Aldrich, catalog number: B4501-1G)
 - Calcium pantothenate (Sigma-Aldrich, catalog number: 21210-25G-F)
 - b. Vitamins and trace elements (Vitamix)
 - Folic acid (Fisher Scientific, catalog number: BP251910)
 - Inositol (Sigma-Aldrich, catalog number: 57569-25G)
 - Niacin (Acros Organics, catalog number: 128291000)
 - P-aminobenzoic acid (Sigma-Aldrich, catalog number: A9878-25G)
 - Pyridoxine HCl (Acros Organics, catalog number: 150770500)
 - Riboflavin (Sigma-Aldrich, catalog number: R9504-25G)
 - Thiamine HCl (Sigma-Aldrich, catalog number: T4625-25G)
 - Boric acid (Sigma-Aldrich, catalog number: B6768-500G)
 - Copper sulfate (Sigma-Aldrich, catalog number: C1297-100G)
 - Potassium iodide (Avantor Performance Materials, catalog number: JT3168-4)
 - Ferric chloride (Acros Organics, catalog number: 217091000)
 - Manganese sulfate (Sigma-Aldrich, catalog number: M7634-100G)
 - Sodium molybdate 2 (Sigma-Aldrich, catalog number: 243655-5G)
 - Zinc sulfate (Sigma-Aldrich, catalog number: Z4750-100G)

- c. Complete Supplement Mixture (CSM)
 - Adenine (Sigma-Aldrich, catalog number: A9126-25G)
 - Arginine (Sigma-Aldrich, catalog number: A5131-100G)
 - Aspartic acid (Acros Organics, catalog number: 105041000)
 - Histidine (Sigma-Aldrich, catalog number: H8000-25G)
 - Isoleucine (Acros Organics, catalog number: 166170250)
 - Leucine (Sigma-Aldrich, catalog number: L8000-100G)
 - Lysine (Sigma-Aldrich, catalog number: L5626-100G)
 - Methionine (Sigma-Aldrich, catalog number: M9625-25G)
 - Phenylalanine (Acros Organics, catalog number: 130311000)
 - Threonine (Acros Organics, catalog number: 138930250)
 - Tryptophan (Acros Organics, catalog number: 140590250)
 - Tyrosine (Acros Organics, catalog number: 140641000)
 - Uracil (Acros Organics, catalog number: 157300250)
 - Valine (Acros Organics, catalog number: 140811000)
- d. Bacto-agar (BD, catalog number: 214030)
- 25. Buffer B (see Recipes)
 - Sorbitol (Sigma-Aldrich, catalog number: S6021-1KG)
 - Potassium phosphate (dibasic) (Sigma-Aldrich, catalog number: P3786-500G)
- 26. Spheroplasting Buffer (see Recipes)
 - Vanadyl ribonucleoside complex (Fisher Scientific, catalog number: 50-812-650)
- 27. Hybridization Buffer (see Recipes)
 - Dextran sulfate (Sigma-Aldrich, catalog number: D8906-10G)
 - Escherichia coli* tRNA (Sigma-Aldrich, catalog number: R1753-500UN)
 - BSA (RNase free) (Fisher Scientific, catalog number: BP671-1)
 - 20x SSC (RNase free) (Thermo Fisher Scientific, catalog number: AM9763)
- 28. Wash Buffer (see Recipes)
 - Formamide (RNase free) (VWR, catalog number: 97061-392)
- 29. Imaging Buffer (see Recipes)
 - 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Sigma-Aldrich, catalog number: 238813-1G)
 - Tris base (For making 200 mM, pH 8 Tris- HCl) (Fisher Scientific, catalog number: BP152-500)
 - Protocatechuic acid (PCA) (Sigma-Aldrich, catalog number: 08992-50MG)
 - Protocatechuate-3,4-dioxygenase (PCD) (Sigma-Aldrich, catalog number: P8279-25UN)

Equipment

1. Pipettors (e.g., VWR, catalog number: 75786-304)
2. x-y translation mount (Thorlabs, catalog number: ST1XY-S)

3. Fiberport (Thorlabs, catalog number: PAF-X-11-PC-A)
Note: This product has been superseded by part number PAF2P-11A.
4. Fiber optic cable (Thorlabs, catalog number: SM450)
5. Single mode fiberoptic cable (Thorlabs, catalog number: P5-460B-PCAPC-1)
6. Widefield microscope (e.g., Olympus, model: IX81)
7. Spectrophotometer (e.g., Eppendorf, catalog number: 2231000516)
8. Centrifuge (e.g., Thermo Fisher Scientific, model: Sorvall™ Legend™ XTR, catalog number: 75004521)
9. Lenses (e.g., Thorlabs, catalog numbers: ACN127-020-A, LB1157-A)
10. Filters and dichroics (e.g., Semrock, catalog numbers: BLP01-635R-25, FF650-Di01-25x36)
11. Micro-centrifuge (Eppendorf, catalog number: 022620100)
12. Incubator (e.g., Thermo Fisher Scientific, catalog number: 50125590)
13. Autoclave (e.g., YAMATO SCIENTIFIC, catalog number: SM300)
14. 60x or 100x high NA objective (e.g., Olympus, UPlanSApo 100X/1.4 Oil)
15. EMCCD camera (e.g., Andor Technology, model: iXon^{EM} +)
16. Laser illumination (e.g., solid state laser: Oxxius, model: LCX-532L-100; Coherent, catalog number: 1185055)
17. Slide translation stage (e.g., Ludl Electronic Products, model: BioPoint2 X-Y Stage)

Software

1. Melting temperature calculator (IDT, <http://www.idtdna.com/calc/analyzer>)
2. RNA folding calculator (Mfold, <http://unafold.rna.albany.edu/?q=mfold>)
3. Sequence specificity check (BLAST, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>)
4. Microscope control (Micromanager, <https://micro-manager.org/>)
5. Spot counting software (Fish-Quant, https://bitbucket.org/muellerflorian/fish_quant)
6. Matlab

Procedure

A. Probe design

DNA oligo probes are designed by selecting an 18-30 nucleotide region in the target mRNA using four criteria.

1. The choice of probe length should be determined by the RNA-DNA melting temperature of the sequence, which can be done by using the calculator provided on the Integrated DNA Technologies, Inc (IDT) website.
2. The probe should have minimal secondary structure.

3. The target sequence should have minimal secondary structure. The secondary structure can be calculated for the RNA by using the Mfold website (Zuker, 2003). A proper choice has the 5' end of the probe binding inside a loop rather than a stem in the secondary structure of the mRNA.
4. The probe should be checked for homology with other sequences in the genome using BLAST (Basic Local Alignment Search Tool) (Johnson *et al.*, 2008).

Choice of fluorophore is dictated by the illumination scheme. Best results were obtained using Cy3 and Cy5 dyes labeled internally (available through IDT). Other labeling schemes such as end labeling work adequately, but are less photostable. In budding yeast, cellular auto-fluorescence is high in the same region of the emission spectrum as dyes excited by 470 nm or 488 nm light (Atto-488 and Alexa 488) and these dyes are not recommended.

When labeled internally the fluorophore should be positioned 2 nt away from either end.

B. Microscope design

Our microscope is a custom-built microscope configured in highly inclined illumination geometry (HILO). For single fluorophores, epi-fluorescence will not generate an adequate signal, Figure 1. It is necessary to have a variable angle geometry on the illumination (most TIR/HILO microscopes have this.) This can be achieved by using a fiber coupled laser or coupling a free space laser to a fiber using a fiberport and a fiber optic cable and moving the fiber output with an x-y translation mount (see Figure 2). Using the x-y translation, the beam can be adjusted away from the center of the back focal plane of the objective. Once this beam is displaced to the critical angle, total internal reflection geometry is achieved. The angle for inclined illumination is less than the critical angle and should be selected for optimum z-sectioning and intensity in the volume of interest.

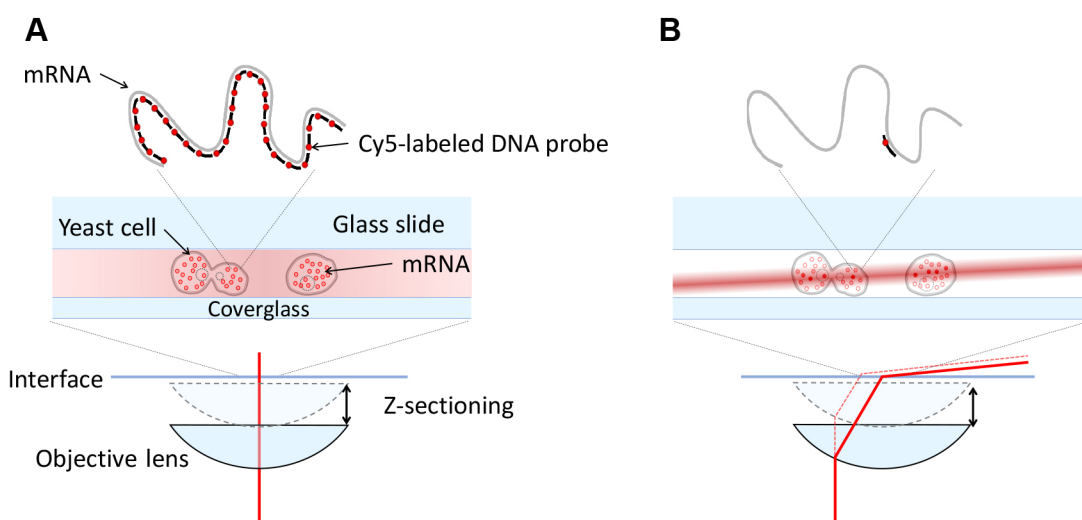


Figure 1. Comparing Epi and HILO illumination. A. An epi-fluorescence microscope has the illumination incident (red line) on the sample through the objective and the entire volume of the sample is illuminated. This leads to poor signal to noise for single fluorophores since the widefield microscope collects out of focus light. B. An inclined illumination geometry has the illumination (red line) displaced radially in the back focal plane of the objective so that the light

becomes a thin laminated sheet in the sample volume (Tokunaga *et al.*, 2008). This enables z-sectioning and increases signal to noise significantly.

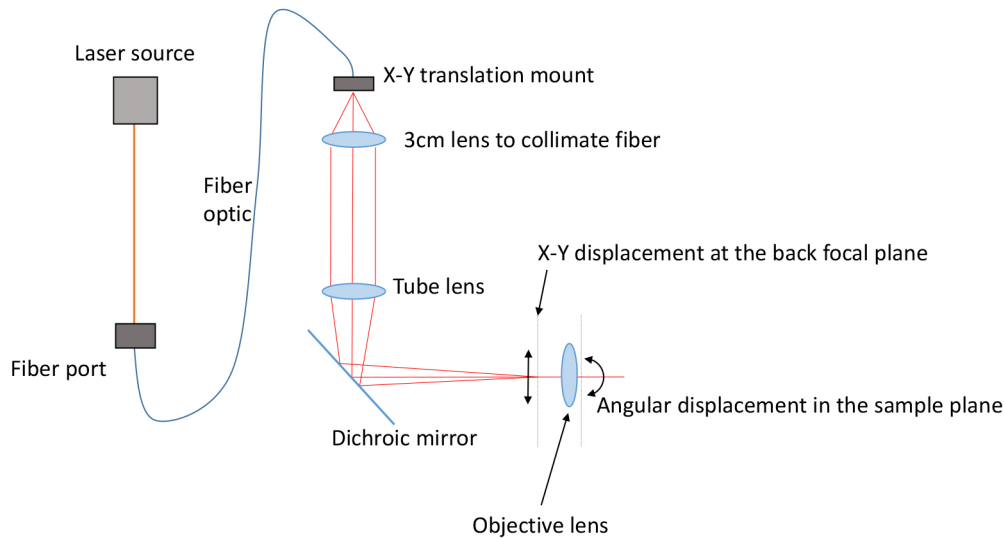


Figure 2. Schematic of the illumination path. One or more lasers are coupled into a fiber optic cable using a fiberport. This fiber is fitted onto an X-Y translation mount and collimated by a short lens (focal length chosen by the field of view). The tube lens focuses the illumination on the back focal plane. When the X-Y position is adjusted away from the center of the back focal plane the beam becomes inclined through the sample volume.

C. Sample preparation

Day 1

At the end of the day, inoculate yeast cells into 50 ml of liquid media in a culture flask from cells actively growing on a plate.

Day 2–Cell fixation and permeabilization

1. Measure Cell OD using a spectrophotometer at OD₆₀₀ by placing 1 ml of cell culture in a cuvet.
2. Once cell OD₆₀₀ has reached 0.6, decant cells into a 50 ml Falcon tube and pelleted by centrifuging at 671 x g for 5 min and aspirated.
3. Resuspend the pellet in 10 ml of ice cold (4 °C) methanol for 10 min for fixation.
4. Cells are pelleted and resuspended in ice cold Buffer B twice and aspirated.
5. Resuspend the cells in 1 ml of Spheroplasting buffer and transfer to a 1.75 ml microcentrifuge tube and add 2 µl of 5 units/µl of zymolyase and gently pipette to mix.
6. Incubate the cells for 30 min or until the OD₆₀₀ of 100 µl of cells added to 900 µl of deionized water shows a reduction of 30% from the initial OD after 1 min, which demonstrates cell lysis.
7. Pellet the cells and aspirate. Centrifuge at no more than 268 x g since they are fragile once they are spheroplasts.

8. Wash the cells two more times in ice cold Buffer B and aspirate (wash means spin down to a pellet, aspirate liquid and resuspend.)
9. Resuspend the cells in 1 ml of 70% ethanol and keep at 4 °C for a minimum of 1 h to overnight.

Day 2 cont./Day 3—hybridization

1. Pellet cells at 268 x g and wash twice each with 1 ml Wash Buffer and aspirate.
Note: Wash buffer should be prepared fresh and formamide should be warmed to room temperature before opening.
2. Dilute the probes to 1 μM in 10 mM Tris-HCl, pH 8.
3. Prepare a mixture of hybridization buffer and probes based on the working concentration determined by titration (see Notes).
4. Resuspend cells to a final volume of 100 μl in the probe-hybridization buffer mixture by gentle pipetting.
5. Then wrap samples with aluminum foil and place in the incubator at 30 °C overnight.

Day 3/Day4—Slide Preparation

1. Prepare Imaging Buffer immediately before use.
2. Wipe the slides with ethanol; or (optional) clean slides and coverslips in a plasma cleaner for 10 min.
Note: The slide should be clean of dust and other particles by wiping with ethanol. Any air bubbles will severely impact the performance of the Imaging Buffer.
3. Mix 2.5 μl of Imaging Buffer with 2.5 μl of cells and place on the coverslip.
4. Place the coverslip on a slide and seal with epoxy along the edges.
Note: Slides should be kept in a dark place while not on the microscope. Several slides can be prepared simultaneously. Once sealed, the performance of the imaging buffer will not degrade for several hours.

D. Data acquisition

Using a microscope as described above, hardware control and the acquisition parameters can be set in the Micromanager software (Edelstein *et al.*, 2014). Z-stack images can be acquired using the multi-dimensional tool in Micromanager for each channel of interest (e.g., DIC, Cy5, *etc.*).

1. Acquire images at full region of interest (512 x 512) on an EMCCD (iXon^{EM} +, Andor) at 100 msec exposure times. The pixel size and z-step are chosen to be less than or equal to the Nyquist sampling limit for the shortest wavelength (see Introduction to Fourier Optics 3rd edition, Goodman.).
2. The Nyquist limit for a widefield microscope is shown by the following equations where the wavelength $\lambda_{emission}$ used is the peak emission of the fluorophore chosen, the angle θ is the half-aperture angle, and n is the index of refraction of the immersion oil.

$$Pixel_{xy} = \frac{\lambda_{emission}}{4n\sin\theta} \quad (1)$$

$$Pixel_z = \frac{\lambda_{emission}}{2n(1-\cos\theta)} \quad (2)$$

$$NA = n\sin\theta \quad (3)$$

These values allow the maximum amount of spatial information to be recorded. Sampling above this limit is acceptable although the resolution between two objects will not be improved. Undersampling will lead to the inability to distinguish between two close fluorophores because the information in one pixel is not coupled to the information in its neighboring pixels.

3. Set the laser to output 25 mW at the sample plane. For each chamber, the thickness is approximately 2 μm and an appropriate number of z-slices are acquired. An example of the contrast between a negative control and a low copy number strain is shown in Figure 3.

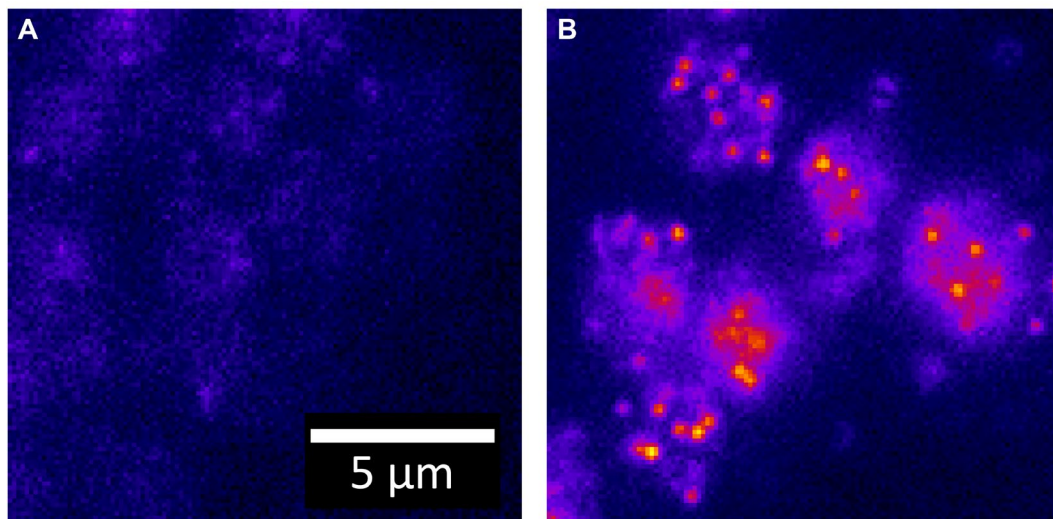


Figure 3. Contrast between strains. A. One z-slice of a negative control for yEvenus mRNA (e.g., wildtype) is shown. All intensity is due to auto-fluorescence. The yEvenus probes are 26nt in length and labeled internally (sequence in Wadsworth, *et al.*, 2017). B. One z-slice of a strain expressing a low copy number (< 20) of yEvenus mRNA transcripts per cell is shown. mRNA transcripts are targeted with a single Cy5 labeled DNA oligo probe.

Data analysis

The Matlab Image Processing Toolbox was used to analyze the three-dimensional images. In cases where the researcher is unfamiliar with coding, we recommend FISH-quant for its rigor and user-friendly GUI. For systems with very non-uniform illumination, Corrected Intensity Distributions using Regularized Energy minimization (CIDRE) (Smith *et al.*, 2015) can be used to flatten the images. Many functions in the Image Processing Toolbox can be accelerated by simply converting them to a `gpuArray` () in Matlab with a compatible graphics card (e.g., Nvidia Geforce 1080). An outline of the algorithm used to locate cells and spots is as follows:

A. Segmentation

1. Perform edge detection on the sharpest DIC image using the Sobel filter in Matlab.
2. Connect detected edges using 1 x 4 and 4 x 1 structural elements.
3. Perform binary morphological erosion and dilation of the image.
4. Label the detected regions in the binary image using the `bwlabeln ()` function.

B. Spot detection

1. Apply a wide Gaussian filter to the image and subtract the result from the raw data as an approximation of background fluorescence.
2. Apply a Laplacian of Gaussian (LoG) filter to the result to enhance the spots.
3. Inside each region detected by segmentation, find pixels that are local maxima in the LoG result and call those spot candidates.
4. Fit each spot candidate with a Gaussian profile and subtract the mean intensity of the annular region around the spot from each pixel included in the spot to get the spot intensity.
5. Determine some threshold brightness based on the intensity of single spots detected on the surface of the slide.
6. Count the spots detected in each cell by using logical indexing of the segmented cells on an 3D array where the detected centers are marked as 1 and all other locations are 0.

Notes

A. Probe design notes

We have used single probes of lengths between 18-30 nt with both internal labeling and end labeling. When compared to a standard smFISH probe set (Biosearch Technologies), we found that the probes hybridized between 50-70% of the true transcript count (Wadsworth *et al.*, 2017). This seems to have more dependence on melting temperature than length. Further, we found that the modified Cy5 analog Quasar 670 was much dimmer (it has a smaller molar extinction coefficient) so that Cy5 outperformed substantially. Finally, when using single probes that can be added independently of one another, we found that the true transcript count was achieved with 4-5 probes (Wadsworth *et al.*, 2017).

B. Microscope

It is not necessary to have a custom-built microscope. The minimum criteria for a microscope to detect single fluorophores is a ~1.4 NA 100X Objective, an EMCCD, a coherent light source with at least 5 mW of power at the sample plane, and variable angle illumination (TIR/HILO.) Any microscope that can be adjusted from epi-fluorescence to total internal reflection geometry is adequate to accomplish HILO. We do not recommend TIR geometry as the yeast samples are general 2-3 μm thick and this is well outside the range of TIR. TIR will only illuminate probes non-specifically bound to the surface and not in the cytoplasm.

We do not recommend a confocal microscope because the signal is in the 1-2 photon limit that is beyond the capability of most turnkey confocal setups. Also, we do not recommend sCMOS or CCD cameras because of the 1-2 photon regime. We have tested several in-house and had poor results.

With the Andor EMCCD camera (EM stands for electron multiplying), we can observe single fluorophores in either a HILO or total internal reflection geometry with nominal gain that is between 50-500 depending on the fluorophore. With our illumination setup, we find no reason to expose for longer than 100 msec although exposures of 1 sec to 2 sec are common in literature.

C. Sample Preparation: Day 2 notes

1. Spheroplasting is the most critical step in order to get the probes inside the cell. If the population of cells is poorly converted to spheroplasts, there will be a lot of variability in the FISH spots counted. Spheroplasting can also be observed by DIC imaging. The cells should appear more round and there should be cell wall debris in the sample.
2. While overnight treatment with ethanol is acceptable, we find the most consistent results when cells are hybridized after 1 h of ethanol treatment.

D. Day 2cont./Day 3 – hybridization notes

1. When working with a sample in hybridization buffer, a sample volume that is too small will not produce a large enough pellet after hybridization. This is particularly noteworthy when trying to work with initial cell culture volumes of 5 ml to 10 ml. Also, it should be noted that the pellet should be around 10 μ l to 20 μ l of the hybridization volume. Larger concentrations of cells cause poor efficiency of probe penetration in any dense clusters of cells.
2. Create serial dilutions of probes in hybridization buffer from 1 nM to 100 nM or a range where the detected number of FISH spots in the sample plateaus. This concentration is chosen as the working concentration of probes. 65 nM working concentration probes seems to be appropriate for probes of 60 °C RNA-DNA melting temperature in the range of 18-26 nt.
3. When imaging, if there are many probes diffusing in solution around your cells, then the cells need further washing to remove unbound probes.
4. Commercial anti-fade reagents such as Prolong Gold may work, but have not been tested in our lab for single fluorophore detection.

E. Day 3/Day4 – Slide preparation notes

The imaging buffer will not perform well if not used immediately. When imaging, if probes rapidly photobleach as the slide is explored by moving from one field of view to the next with live viewing, which means your imaging buffer is not working due to expiring or due to tiny bubbles in the chamber (they can be seen by eye).

We avoid using nail polish although it is common, because it contains solvents that will wick into the sample and cause photobleaching or auto-fluorescence. We find epoxy does not have that effect.

F. Data acquisition notes

1. We use a 1.4 NA 100x objective with additional magnification to oversample based on the Nyquist criterion. The camera is set to Frame Transfer mode. The hardware binning is set to 1 x 1. The gain is set to a level that confines the intensity to the dynamic range of the camera on the brightest sample (200 for Cy5).
2. When trouble shooting poor signal to noise, there are several potential sources of auto-fluorescence; methanol, ethanol, and formamide. We found that lower grade methanol and ethanol are fluorescent in the visible spectrum and use \leq 99.5 ACS grade reagents. Also, if the auto-fluorescence appears during the hybridization procedure, it is likely because of formamide being contaminated. Formamide is very delicate and will convert to formic acid when mixed with water from condensation if handled poorly. This will lead to inconsistent results.

Recipes

Q: The concentration at the end of reagent.

1. SD Complete (1 L), pH 5.8
 - Carbon, Nitrogen, and Salts (CNS) 26.7 g
 - Dextrose 20 g
 - Ammonium sulfate 5 g
 - Potassium phosphate monobasic 1 g
 - Magnesium sulfate 0.5 g
 - Sodium chloride 0.1 g
 - Calcium chloride 0.1 g
 - a. Vitamins and trace elements (Vitamix) 2,840 μ l

Biotin (5 mg/50 ml)	20 μ l
Calcium pantothenate (1 g/50 ml)	20 μ l
Folic acid (5 mg/50 ml)	20 μ l
Inositol (0.5 g/50 ml)	200 μ l
Niacin (0.1 g/50 ml)	200 μ l
P-aminobenzoic acid (50 mg/50 ml)	200 μ l
Pyridoxine HCl (1 g/50 ml)	20 μ l
Riboflavin (5 mg/50 ml)	2 ml
Thiamine HCl (1 g/50 ml)	20 μ l
Boric acid (1.25 g/50 ml)	20 μ l
Copper sulfate (0.1 g/50 ml)	20 μ l
Potassium iodide (0.25 g/50 ml)	20 μ l
Ferric chloride (0.5 g/50 ml)	20 μ l
Manganese sulfate (1 g/50 ml)	20 μ l
Sodium molybdate (0.5 g/50 ml)	20 μ l

- Zinc sulfate (1 g/50 ml) 20 μ l
- b. Complete Supplement Mixture (CSM) 790 mg
 - Adenine 10 mg
 - Arginine 50 mg
 - Aspartic acid 80 mg
 - Histidine 20 mg
 - Isoleucine 50 mg
 - Leucine 100 mg
 - Lysine 50 mg
 - Methionine 20 mg
 - Phenylalanine 50 mg
 - Threonine 100 mg
 - Tryptophan 50 mg
 - Tyrosine 50 mg
 - Uracil 20 mg
 - Valine 140 mg
- c. Bacto-agar 20 g optional
- 2. Buffer B (1 L)
 - Sorbitol (218 g)
 - Potassium phosphate (dibasic) (17.4 g)
 - RNase free water
- 3. Spheroplasting Buffer (10.1 ml)
 - Buffer B 10 ml
 - Vanadyl ribonucleoside complex (200 mM) 100 μ l
- 4. Hybridization Buffer (10 ml)
 - Dextran sulfate 1 g
 - Escherichia coli* tRNA 10 mg
 - Vanadyl ribonucleoside complex (200 mM) 100 μ l
 - BSA 40 μ l (5 mg/ml) (RNase free)
 - 20x SSC 1 ml (RNase free)
 - RNase free water
- 5. Wash Buffer (50 ml)
 - Formamide 5 ml (RNase free)
 - 20x SSC 5 ml (RNase free)
 - RNase free water
- 6. Imaging Buffer (100 μ l)
 - Trolox (1 mM) 70 μ l
 - 20x SSC 10 μ l
 - Tris-HCl (200 mM, pH 8) 5 μ l

Protocatechuic acid (PCA) (25 mM) 10 μ l
Protocatechuate-3,4-dioxygenase (PCD)(200 nM) 5 μ l

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Competing interests

The authors declare no conflicts of interests or competing interests.

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