# Single-mRNA counting using fluorescent *in situ* hybridization in budding yeast

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**Fluorescent *in situ* hybridization (FISH) allows the quantification of single mRNAs in budding yeast using fluorescently labeled single-stranded DNA probes, a wide-field epifluorescence microscope and a spot-detection algorithm. Fixed yeast cells are attached to coverslips and hybridized with a mixture of FISH probes, each conjugated to several fluorescent dyes. Images of cells are acquired in 3D and maximally projected for single-molecule analysis. Diffraction-limited labeled mRNAs are observed as bright fluorescent spots and can be quantified using a spot-detection algorithm. FISH preserves the spatial distribution of cellular RNA distribution within the cell and the stochastic fluctuations in individual cells that can lead to phenotypic differences within a clonal population. This information, however, is lost if the RNA content is measured on a population of cells by using reverse transcriptase PCR, microarrays or high-throughput sequencing. The FISH procedure and image acquisition described here can be completed in 3 d.**



## INTRODUCTION

FISH is the method of choice for detecting specific nucleic acids in their native cellular environment. Since the first application of fluorescent probes for *in situ* detection of RNA[1], the technique has been improved and modified for a wide range of targets and probing methods (reviewed in refs. 2–4); however, its basic principles have remained unchanged since its inception almost 30 years ago. Its ability to detect transcripts *in situ* while preserving cellular spatial and temporal information led to the discovery of β-actin mRNA localization to the lamellipodia in chicken embryonic myoblasts and fibroblasts[5] and of *ASH1* mRNA localization to the mitotic bud in *Saccharomyces cerevisiae*[6].

Fluorescence-based detection of RNAs was developed as an alternative to radiolabeled-probe[7] methods and their several drawbacks, such as the handling of hazardous material and loss of activity because of isotopic decay. The earliest detection of an mRNA using fluorescence occurred in 1982, when a target mRNA was hybridized with a nick-translated, biotinylated probes and detected with fluorescently labeled streptavidin conjugates[1]. Within the next 10 years, this approach further evolved to the system that is still in use today, which uses synthetic, single-stranded, antisense DNA probes labeled with enough fluorescent dyes to allow direct detection of a target mRNA[8]. Further advancements in microscopy equipment and detection algorithms allowed FISH to become quantitative. The analysis of individual RNAs hybridized with a single FISH probe provided the first single-molecule, single-cell counting of an mRNA with high spatial resolution and enabled the analysis of nascent chain dynamics at the site of transcription of an endogenous gene[9]. Since then, the FISH protocol has been modified to accommodate the labeling of an mRNA with multiple fluorescent probes, a step that strongly increased the signal-to-noise ratio and therefore detection sensitivity. With further technological advances in fluorescence microscopy and development of algorithms enabling high-throughput image analysis, FISH now permits simultaneous visualization of multiple mRNA targets or mRNA regions by using FISH probes that are labeled with spectrally distinct fluorophores[10–12].

### Applications of the method

Since 1990, FISH has been increasingly applied to a variety of organisms and biological systems, propelled mostly by the development of techniques providing simple probe synthesis and FISH signal detection[3]. FISH can be implemented to characterize all the steps in the mRNA life cycle, including transcription, splicing, export, translation/localization and decay. Specifically, in budding yeast, single-molecule FISH has been implemented to investigate various biological processes, such as the stochastic nature of transcription[12], coordinated transcription in protein complex formation[11], transcriptional oscillations controlling the metabolic cycling[13], spatiotemporal coordination of mitochondrial biogenesis[14] and mRNA localization[6] and regulation of mRNA decay during the cell cycle[15]. Multicolor FISH enabled calculation of distribution of RNA polymerase II (RNAPII) complexes on a transcribing gene and provided a direct measure of transcriptional activity, RNAPII velocity and 3′ end termination time during nascent chain synthesis[9,12]. In the mouse intestine, multicolor FISH has allowed the determination of molecular markers in adult stem cells[16]. Finally, numerical fitting of a FISH-labeled diffraction-limited mRNA can also provide the cellular position of the particle with subpixel resolution[17]. Single-molecule FISH, therefore, enables the determination of the spatial location of individual transcripts within a cell and relative to any given cellular structure.

### Experimental design

**Overview of the procedure.** Here we describe a single-molecule FISH protocol suitable for detection of transcripts in *S. cerevisiae*. It is based on the use of four to ten single-stranded, antisense DNA probes, each of which is about 50 nt long and labeled with four or five fluorescent dyes. Probes are either synthesized in the laboratory with a DNA/RNA synthesizer[18] or are commercially available (Biosearch Technologies) and labeled with a fluorescent dye, such as Cy3, Cy3.5 or Cy5. After fixation, the yeast cell wall is removed with lyticase to permeabilize the cells for subsequent probe hybridization. Hybridization is carried out in 40% (wt/vol) formamide at 37 °C.

This step 'loosens' the protein-RNA interactions and enables the probe to access to the mRNA; it also decreases nonspecific probe binding with nontarget mRNAs and with the cellular background. The hybridized cells are imaged in three dimensions using a wide-field epifluorescence microscope equipped with a mercury or metal-halide lamp, a high-numerical-aperture objective to maximize the collection of emitted photons, a CCD camera, and filter sets optimized to maximize emission collection and minimize spectral overlap (in the case of multicolor FISH). Because nonspecific binding of individual FISH probes cannot be entirely prevented, the iteration of the probes on the target mRNA is the basis for the increased signal occurring within a diffraction-limited volume. Because of this the signal-to-noise ratio, numerical fitting of this diffraction-limited spot can yield accurate quantification of the fluorescence intensity of the particle[17]. Images of FISH-hybridized yeast cells are acquired in three dimensions with a wide-field epifluorescence microscope and then maximally projected using the ImageJ 3D Projection Volume Rendering plug-in. With a spot-detection algorithm called Localize, FISH-labeled mRNAs are detected and counted. Cells are manually segmented with an ImageJ segmentation plug-in and a number of mRNAs per cell are determined. Before being maximally projected and analyzed by Localize, images can be deconvolved with the Huygens program to enhance the signal-to-noise ratio of FISH-labeled mRNA relative to the cellular background.

**Single-molecule detection algorithm (Localize).** FISH images in yeast consist of individual fluorescent punctae dispersed throughout the cytosol and the nucleus, necessitating an automated spot-detection algorithm capable of measuring the number, positions and intensities of the punctae. Each spot corresponds to the diffraction-limited image of a subresolution object (object with a size less than the resolution of the light microscope). Therefore, the spatial extent of the spot, referred to as the point spread function, is determined by the properties of the microscope, not the size of the RNA. Spot segmentation can then be specifically adapted to the shape of the point spread function, which closely resembles a 2D Gaussian[17]. We use an implementation of an algorithm known as the Gaussian mask algorithm, which relies on an iterative fit to a 2D discrete Gaussian[17,19]. This algorithm performs well in the presence of background and autofluorescence and is easy to implement. The software we have developed in the IDL programming language (ITT Visual Information Systems) for FISH image quantification proceeds through the following steps: (i) spatial band-pass filtering to identify candidate spots for further analysis, (ii) local background subtraction to remove any residual offset or unevenness in the image, (iii) Gaussian mask fitting to find the center and the intensity of each spot and (iv) culling of spots on the basis of duplication and/or an intensity threshold. Spot intensities and positions are written to a data file for further analysis[12,19]. As intensity is usually the crucial parameter for differentiating signal from background, care must be taken in any algorithm to ensure the best possible recovery of the spot intensity from the image. The Gaussian mask has the benefit of iterating between the ideal shape of the point spread function and the actual data to determine the intensity, rather than using a single value (e.g., the amplitude of a Gaussian fit) to determine the total intensity.

**ImageJ plug-in for cell segmentation.** This plug-in was written in the Singer laboratory and is used to manually segment yeast cells in ImageJ. With a graphic tablet or a mouse, the plug-in creates a cellular boundary and records the image coordinates of this boundary. These coordinates then determine which FISH-labeled mRNAs segment into a particular yeast cell.

**Huygens.** Huygens is an image restoration program from Scientific Volume Imaging based on various deconvolution algorithms that allow the recovery of objects from images that are degraded by blurring and noise. By deconvolving an image, the signal-to-noise ratio of a fluorescently labeled mRNA can increase, which can ease subsequent single-molecule detection. For a detailed description of Huygens, see the manufacturer's instructions. The use of Huygens is optional during single-molecule detection.

**Controls.** Single FISH probes hybridize specifically to the target mRNA as well as nonspecifically to the cellular background. The use of multiple probes that are efficiently labeled and that hybridize efficiently to the mRNA is necessary to distinguish between specific and nonspecific FISH signal. During the single-mRNA analysis, we take advantage of the nonspecific cellular probe binding and use it to calibrate the fluorescence intensity of a single mRNA by the number of single probes used in a FISH probe mixture. We have found that the fluorescence intensity of a single mRNA labeled with a mixture of probes equals the fluorescence intensity of a single probe nonspecifically hybridizing to the cellular background multiplied by the number of probes used to label the mRNA. This relationship indicates that probes hybridize efficiently and further validates the identity of the detected spots as single mRNAs.

It is important to further validate the identity of detected fluorescent spots as single mRNAs by performing one of several available controls. When the background cellular fluorescence is high, either because of nonspecific single FISH probe binding or because of cellular autofluorescence, increasing the number of FISH probes in a probe mixture increases the fluorescence intensity of the FISH-labeled mRNAs, which eases their detection. In budding yeast, at least four probes that anneal to different parts of an mRNA are used in a mixture. It is important that these probes are also efficiently labeled, as probes that are coupled to multiple fluorescent dyes also increase the fluorescent brightness of a FISH-labeled mRNA and ease subsequent single-mRNA detection. After the FISH probes are coupled to the fluorescent dye, this labeling efficiency can be calculated according to Beer-Lambert's law. Probes with a labeling efficiency higher than 90% are typically used for single-mRNA counting.

A good way of determining whether the FISH signal is specific or not is to observe whether changes in mRNA profiles occur if the expression patterns of a gene are perturbed, either genetically or chemically. For example, the deletion of a gene will result in the loss of a specific fluorescent signal, and the inhibition of transcription will cause a gradual depletion of FISH-labeled transcripts over time as a result of mRNA decay, whereas the deletion of a protein that regulates cellular mRNA localization will result in the deregulation of mRNA localization patterns. Finally, colocalization of spectrally distinct fluorescent signals targeted to the same mRNA in a two-color FISH provides further controls for single-molecule FISH. For example, if the 5′ end of the mRNA is hybridized with a mixture of FISH probes coupled to the Cy3 dyes while the 3′ end of an mRNA is hybridized with a mixture of FISH probes coupled to the Cy3.5 dyes, labeled mRNAs will have colocalized Cy3 and Cy3.5 FISH signals.

### Limitations

Because the detection of single mRNAs in a 3D image is computationally intense, a maximally projected image is analyzed instead. As a consequence, spots with similar fluorescence intensities found in pixels directly above or below each other will maximally project as a single spot without the summation of the fluorescent signal. This will lead to undercounting of mRNAs when a highly expressed or a localizing mRNA is analyzed. The counting protocol described here is thus optimized for mRNAs of low or moderate expression with up to approximately 30–40 mRNAs per cell.

In the age of the genome-wide transcriptome analysis enabled by microarrays and high-throughput sequencing, the inability to analyze a large number of mRNAs simultaneously and rapidly represents a drawback of a single-molecule FISH. To date, multicolor FISH has enabled the simultaneous detection of only up to three mRNA species in the cytoplasm[11,20]. This number is restricted mainly by the availability of spectrally distinct bright and photostable fluorescent dyes (e.g., Cy3, Cy3.5 and Cy5) and by the number of filter cubes a microscope can accommodate (e.g., six in an Olympus BX61 upright epifluorescence microscope). In principle, this number could increase if a barcoding approach were used; this is a technique that enabled the simultaneous quantification of transcription site activity of 11 different genes within the same cell[10]. Four spectrally distinct fluorescent dyes can be coupled with FISH probes; however, the number of uniquely labeled mRNAs can increase when a specific color combination is used to determine the identity of the transcript. Beyond the detection of the transcription site activity, this approach has not been used to detect single mRNAs.

### Comparisons with other methods

A variant of the approach described here uses shorter, ~20-bases-long DNA probes each covalently coupled to a single fluorescent dye, typically via the 3′-amine modification. To increase the brightness of the fluorescently labeled mRNA, 48 of these probes per mRNA are used[20–22]. Because shorter probes are used, this protocol necessitates lower formamide concentrations for optimal hybridization between an mRNA and a FISH probe. These probes are commercially available and several companies provide custom-designed probes (Biosearch Technologies)[20]. The two FISH approaches differ primarily in the probe design (see REAGENT SETUP for further details), whereas the spot-detection routine and yeast fixation and FISH probe hybridization steps do not diverge markedly. The spot-detection algorithm used in ref. 20 also relies on a 3D Gaussian fit that, after background correction, determines the integral brightness of the fluorescent spot. To evaluate the correct threshold, images are iteratively overanalyzed over a set of threshold values and the number of detected spots at each threshold value is then plotted. Typically, a plateau is reached at which the number of detected spots does not change with the increasing threshold, thereby indicating that the fluorescent signals of detected spots are separated from the background. This step validates the choice of a threshold parameter during single-mRNA detection routine[20–22].

An alternative to the single-molecule counting with FISH in fixed cells is imaging of RNAs in living cells using MS2-tagged mRNAs. In contrast with FISH, which provides a single snapshot of an mRNA life-cycle in fixed cells, a live-cell approach enables the detection of a single mRNA with a detailed temporal and spatial resolution, beginning with the synthesis of the nascent mRNA chains. The MS2 system is based on the bacteriophage MS2-derived nucleic sequence, repeated 24 times and typically inserted into the 3′ untranslated region of a gene[23]. In the RNA, this repeated sequence forms a hairpin loop that accommodates binding of the MS2 bacteriophage capsid protein, tagged with a fluorescent protein similar to GFP, thus fluorescently labeling the mRNA. As with FISH, multiplication of MS2-binding sites strongly amplifies the signal-to-noise ratio, thus enabling detection of single mRNAs in living cells. The first implementation of this system determined the kinetics of *ASH1* mRNA localization in budding yeast[23] and since then has been employed in a variety of organisms and biological setups (reviewed in refs. 24,25). Development of alternative labels to MS2, such as PP7, U1A and $\lambda_N$, now provide a possibility of simultaneous imaging of multiple RNAs in living cells in time[26–29]. The major drawback of the MS2 and MS2-like systems is that an engineered mRNA must be created to enable detection of the transcript. These mRNAs are thus not endogenous, and the insertion of the repeats into the 3′ untranslated regions could change the synthesis, translation and/or decay dynamics of the mRNA. Despite the development of additional tags, multicolor imaging in living cells has thus far been demonstrated only in a limited number of cases[30]. Thus, routine live-cell imaging has been restricted to a few mRNA species.

Here we provide a comprehensive single-molecule FISH protocol; we start with the preparation of fixed cells, provide a step-by-step hybridization procedure and finish with the image acquisition and data analysis. Finally, we give a detailed account of the crucial steps in the protocol and provide troubleshooting points that will aid in achieving optimal FISH results.

## MATERIALS

### REAGENTS

- Yeast cells and yeast growth medium (Euroscarf, cat. no. 20000A; BMA 64-1A, MATa; ura3-52; trp1Δ 2; leu2-3,112; his3-11; ade2-1; can1-100)
- HCl (1 N; Fisher Scientific, cat. no. A144-500) **! CAUTION** HCl is corrosive. Wear gloves and handle it in a fume hood to avoid contact with eyes and skin.
- Poly-L-lysine (10×, Sigma, cat. no. P8920)
- Probe-labeling buffer (0.1 M $Na_2CO_3$ carbonate buffer, pH 9.0; Sigma, cat. no. S7795)
- Cy3, Cy3.5 or Cy5 monoreactive dye (GE Healthcare/Amersham, cat. nos. PA23001, PA 23501 and PA25001, respectively)
- Paraformaldehyde (32% (wt/vol), Electron Microscopy Sciences, cat. no. 15714) **! CAUTION** It is a hazardous solution and cross-linking agent. Wear protective gloves and handle it under a fume hood.
- Sorbitol (Sigma, cat. no. S1876)
- $K_2HPO_4$ (1 M, Fisher Scientific, cat. no. P288-500)
- $KH_2PO_4$ (1 M, Sigma, cat. no. P-0662)
- $Na_2HPO_4$ (1 M, Fisher Scientific, cat. no. S373-500)
- $NaH_2PO_4$ (1 M, Fisher Scientific, cat. no. S381-500)
- NaOH (1 M, Sigma, cat. no. S-8045) **! CAUTION** NaOH is a corrosive solution. Wear gloves and handle under the fume hood to avoid eyes and skin damage.
- β-Mercaptoethanol (Sigma, cat. no. M-6250)
- Ribonucleoside vanadyl complex (VRC; New England Biolabs, cat. no. S1402S)
- Lyticase (Sigma; cat. no. L5263)
- Ethanol (70% (vol/vol)), made from 100% (vol/vol) ethanol (Fisher, cat. no. 22-032-103) and distilled $H_2O$
- Saline-sodium citrate (SSC, 20×, Roche, cat. no. 11 666 681 001)
- Deionized formamide (99% (wt/vol), Acros Organics, cat. no. 327235000) **! CAUTION** It is a toxic solution; avoid contact with eyes or skin and also avoid inhalation and ingestion. Wear protective gloves and handle under the fume hood.
- Amino-allyl thymidines (Glen Research, cat. no. 10-1039-05)

- FISH DNA probes (~50 nt long; **Fig. 1**) custom-made in the laboratory—each probe is 50 nt in length; bold **T** indicates modified amino-allyl thymidines; see REAGENT SETUP: *ASH1-7*, 5′-AGGAGC**T**ATTTGCA TGAGAA**T**CCGGACCAGCAGA**T**AATGCATGCAG**T**GGT-3′; *ASH1-8*, 5′-**TT**GGCGACACATTGAGCGAA**T**GAAACGAATAC**TT**CCTAGG ACTTGC**T**GAA-3′; *ASH1-9*, 5′-AG**TT**ACATAGCTGA**T**CTTGCTTTC **TT**GGCGACCACGATGGCC**T**CCAACAG-3′; *ASH1-10*, 5′-TCAA**TT**TC GCAGTTGCG**TT**CGGGGACGAA**TT**CTTTGTCTATTCCAT**T**GGA-3′
- Sheared salmon sperm DNA (Sigma, cat. no. D7656)
- *E. coli* tRNA (Roche, cat. no. 1010951001)
- BSA (Roche, cat. no. 13465434)
- Triton X-100 (0.1% (vol/vol), Fisher Scientific, cat. no. BD 151500)
- PBS (Roche, cat. no. 11 666 789 001)
- 4′,6-Diamidino-2-phenylindole, dilactate (DAPI, dilactate; Sigma, cat. no. D9564-10MG)
- ProLong Gold mounting medium (Invitrogen/Molecular Probes, cat. no. P36934) or any other commercially available mounting medium
- Distilled, deionized water ($ddH_2O$)

**EQUIPMENT**

- Latex gloves (Fisherbrand, cat. no. 11-394-5)
- DNA synthesizer (394 DNA/RNA Synthesizer (Applied Biosystems) or equivalent)
- Shaker (30°, New Brunswick Scientific, cat. no. 200 r.p.m.)
- Spectrophotometer (Beckman DU 640 Spectrophotometer, Beckman)
- Stirring hot plate (Isotemp; Fisher Scientific)
- Pasteur pipette (Fisher, cat. no. 13-678-4A)
- Rubber tubing (Masterflex, cat. no. 6402-24 and Fisher, cat. no. Tygon R-3603)
- Filter flask (1 liter, Pyrexplus; Fisher Scientific, cat. no. 10-180G)
- Bottle stopper (Fisherbrand, cat. no. 14-135N)
- Forceps (Fisher Scientific, cat. no. 08-953E)
- Fume hood (Safeair; Hamilton)
- Chromatography paper (Whatman, cat. no. 3030 917)
- 6-Well tissue culture plates (Becton Dickinson, cat. no. 18273)
- Vortex (Vortex Genie2; Fisher, cat. no. 12-812)
- QIAquick nucleotide removal kit (Qiagen, cat. no. 28304)
- NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies)
- Falcon tubes (50 ml, BLUE MAX 50-ml polypropylene conical tube; Becton Dickinson, cat. no. 352098)
- Centrifuge (Sorvall RT6000; H-1000B Rotor; 2,400*g*)
- Tabletop centrifuge (Eppendorf, cat. no. 5417C)
- Vacufuge (Eppendorf, cat. no. 07-748-15)
- Petri dish (100 mm × 15 mm; Fisherbrand, cat. no. 0875712)
- Falcon tube cap (50, BLUE MAX 50-ml polypropylene conical tube, Becton Dickinson, cat. no. 352098)
- Parafilm (Fisher Scientific, cat. no. 13-274-16)
- Coplin jars (Electron Microscopy Sciences, cat. no. 72242-21)
- Water incubator (37 °C; Polyscience)
- Orbital shaker (Themolyne, Maxi-Mix III, Type 65800)
- Microscope slides (Gold Seal, cat. no. 3051)
- Microscope cover glass (coverslips; Fisherbrand, cat. no. 12-542-B 22×22-1)
- Wide-field epifluorescence microscope equipped with appropriate filters (see EQUIPMENT SETUP)
- Localize (single-molecule detection algorithm created in the Singer laboratory; see Experimental Design). Contact R.H.S. for availability.
- ImageJ plug-in for cell segmentation (created in the Singer laboratory; see Experimental Design). Contact R.H.S. for availability.
- SigmaPlot data analysis and graphing program (Systat Software)
- ImageJ (Java software for image-processing analysis; freely available at: http://rsbweb.nih.gov/ij/)
- Huygens software (Scientific Volume Imaging)
- Graphic tablet (Wacom, cat. no. CTE-440)
- Adobe Photoshop (Adobe)
- Olympus BX61 upright microscope (Olympus)
- Metal-halide lamp (EXFO X-Cite Series 120 PC, Lumen Dynamics Group)
- CoolSNAP HQ CCD camera (Photometrics)
- Metamorph software (Molecular Devices)

**REAGENT SETUP**

**DNA FISH probe synthesis** The probes are made in the laboratory with an Applied Biosystems 394 DNA/RNA Synthesizer. For a detailed description of the probe design and synthesis protocol see ref. 18. In short, a typical FISH probe is ~50 nt long, with four or five amino-allyl thymidines each covalently bonded to a monoreactive NHS-ester fluorescent dye by an esterification reaction (**Fig. 1a**). These thymidines are spaced minimally 10 nt apart to prevent fluorescence quenching. Alternatively, a recently published method uses shorter probes coupled to dyes at the ends[20]. Both shorter and 50-nt-long FISH probes are also commercially available (Biosearch Technologies). ▲ **CRITICAL** If probes are synthesized in the laboratory, the capping of the amine, which reduces labeling efficiency, can be prevented if the oligonucleotides are synthesized with acetyl-protected deoxycytidine and deprotected in 30% (vol/vol) ammonia/40% (vol/vol) methylamine at 65 °C for 15 min.

**Unlabeled FISH probe mixture** A mixture of a minimum of four FISH probes designed to hybridize to different sites along the mRNA is used. An equal amount of each probe (5 µg total in the mixture) is lyophilized. All probe preparations are stored at −20 or at −80 °C for long-term storage. ▲ **CRITICAL** FISH probes can stick nonspecifically to the protein cellular background. The use of multiple FISH probes in a mixture increases the specific fluorescent signal of a single mRNA relative to nonspecific signal of probe background binding. Because of this signal amplification, individual mRNAs appear as bright spots, thereby facilitating the subsequent single-molecule detection.

**Buffer B (1×)** One liter of 1× buffer B contains 1.2 M sorbitol and 100 mM potassium phosphate buffer (pH 7.5) dissolved in $ddH_2O$ up to 1 liter. Filter-sterilize and store at 4 °C for up to 1 year.

**Buffer B (1.4×)** One liter of 1.4× buffer B contains 1.7 M sorbitol and 140 mM potassium phosphate buffer (pH 7.5), dissolved in $ddH_2O$ up to 1 liter. Filter-sterilize and store at 4 °C for up to 1 year.

**Spheroplasting buffer** A volume of 2.5 ml of spheroplasting buffer contains 1.8 ml of 1.4× buffer B, 5 µl of β-mercaptoethanol, 250 µl of 200 mM VRC and 0.39 ml of $ddH_2O$. ▲ **CRITICAL** Freshly prepare the solution before use.

**Hybridization solution** A volume of 100 ml of hybridization solution contains 40 ml of deionized formamide, 10 ml of 20× SSC and 50 ml of $ddH_2O$. ! **CAUTION** Formamide is toxic and should be handled in a fume hood and discarded according to relevant environmental health and safety instructions. Store formamide at 4 °C. ▲ **CRITICAL** Freshly prepare the solution before use.

**Competitor DNA** To make competitor DNA, combine 50 µl of 10 mg $ml^{-1}$ sheared salmon sperm DNA with 50 µl of 10 mg $ml^{-1}$ *E. coli* tRNA (prepared from solid by dissolving 10 mg in 1 ml of $ddH_2O$). Aliquot into 100-µl volumes. Store at −20 °C for several years.

**Solution F** For one coverslip, mix 16 µl of formamide, 2 µl of 100 mM sodium phosphate buffer (pH 7.5) and 2 µl of $ddH_2O$. Scale up if necessary. ▲ **CRITICAL** Freshly prepare the solution before use.

**Solution H** For four coverslips, mix 24.7 µl of $ddH_2O$, 10 µl of 20× SSC, 10 µl of 20 mg $ml^{-1}$ BSA and 5 µl of 200 mM VRC. Scale up if necessary. ▲ **CRITICAL** Freshly prepare the solution before use.

**DAPI** Prepare a 0.5 µg $ml^{-1}$ DAPI solution in 1× PBS. Stir overnight at room temperature (RT; 20–25 °C) in an flask wrapped in aluminum foil. Store at 4 °C for up to 1 year.

**Probe labeling buffer (0.1 M $Na_2CO_3$ buffer, pH 9.0)** To make 100 ml of buffer, weigh 1.06 g of $Na_2CO_3$ and adjust to 100 ml with $ddH_2O$. Adjust the pH to 9.0 with 1 M NaOH. Aliquot into 1-ml samples and store at −20 °C for several years.

**Sodium phosphate buffer (100 mM, pH 7.5)** Combine 80.95 ml of 1 M $Na_2HPO_4$ with 19.05 ml of 1 M $NaH_2PO_4$ and adjust the volume up to 1 liter with $ddH_2O$. Store at RT for several years.

**Potassium phosphate buffer (100 mM, pH 7.5)** Combine 83.4 ml of 1 M $K_2HPO_4$ with 16.6 ml of 1 M $KH_2PO_4$ and adjust the volume up to 1 liter with $ddH_2O$. Store at RT for several years.

**Potassium phosphate buffer (140 mM, pH 7.5)** Combine 116.76 ml of 1 M $K_2HPO_4$ with 23.24 ml of 1 M $KH_2PO_4$ and adjust the volume up to 1 liter with $ddH_2O$. Store at RT for several years.

**EQUIPMENT SETUP**

**Microscope** A standard wide-field epifluorescence microscope can be used to acquire single-molecule FISH images because of the high signal amplification of the probes used in this protocol. We use an Olympus BX61 upright microscope equipped with Nomarski differential interference contrast (DIC) optics and epifluorescence illumination. A mercury lamp or a metal-halide lamp is used for a light source. A microscope objective with a

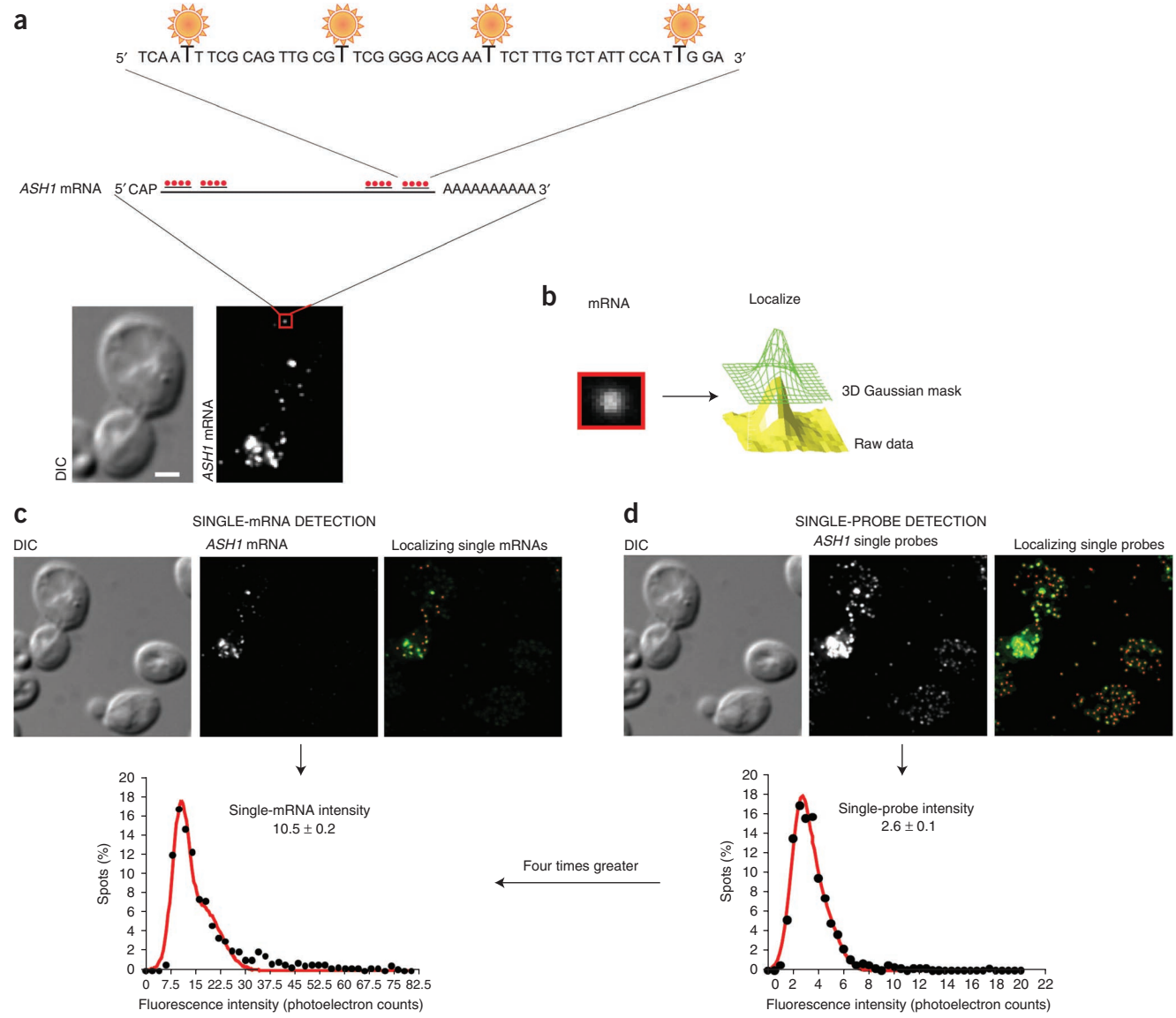


**Figure 1** | Single-mRNA detection and counting. (**a**) A schematic showing *ASH1* mRNA hybridized by four 50-nt-long DNA FISH probes in the open reading frame. A depiction of a 3′-most hybridizing probe with four Cy3-coupled modified thymidines (capital bold T) is shown. (**b**) Calculating integral fluorescence intensity of a spot using Localize. Localize fits a 3D Gaussian mask over each fluorescent spot and determines its total fluorescence intensity. (**c**,**d**) Single *ASH1* mRNA and FISH probe quantification. The culled distribution of integral fluorescence intensities of all spots detected in images (black circles) was fitted to a Gaussian curve (red line) to calculate an average intensity of a single FISH-labeled mRNA or single FISH probe. The average fluorescence intensity of a single mRNA was 10.5 ± 0.2 (photoelectron counts) ($R^2$ = 0.85), four times greater than the average fluorescence intensity of a single probe hybridizing nonspecifically to the cellular background (2.6 ± 0.1 (photoelectron counts), $R^2$ = 0.99). Shown are DIC images of yeast cells, single *ASH1* mRNAs and single *ASH1* FISH probes. Detected single mRNAs and single probes (using Localize) are depicted as red spots. The green background in the localized image corresponds to the FISH-labeled mRNAs. The images were deconvolved with Huygens (Scientific Volume Imaging) before single-molecule analysis. (**e**) Cell segmentation. Yeast cells are segmented using an ImageJ plug-in to obtain an absolute number of mRNAs per cell. Scale bar, 1 µm.

high numerical-aperture (NA) is desirable to collect the maximum number of photons. We typically use either an Olympus PlanApo ×60/1.4 NA or UPlanApo ×100/1.35 NA oil-immersion objective with Olympus Type-F immersion oil (nd 1.516). A standard cooled CCD camera is sufficient to detect single mRNAs using our method. We use a CoolSNAP HQ CCD camera with a pixel size of 6.45 µm. The fluorescence filter sets are optimized to maximize emission collection and minimize the spectral overlap in case of multicolor FISH. We normally use the Chroma filter set 31000 (DAPI), 41007a (Cy3) and 41008 (Cy5). When multiple mRNAs are labeled with Cy3 and Cy3.5 simultaneously, narrow band-pass filters such as the Chroma filter set SP102v1 (Cy3) and SP103v1 (Cy3.5) are used. Images are acquired with Molecular Devices Metamorph software.

**Tabletop aspirator** Place the rubber stopper into the filter flask. Attach the Masterflex tubing on one side to the rubber stopper and to a Pasteur pipette on the other side. Attach the Tygon tube to the filter flask and connect it to the air outlet in the laboratory.

## PROCEDURE

### Growing yeast cells for FISH ● TIMING 48 h (Steps 1–9 can be performed concurrently)

**1|** Grow 50 ml of yeast culture at 30 °C, with shaking at 250 r.p.m.

▲ **CRITICAL STEP** It is important to keep the cells at a low optical density at 600 nm ($OD_{600}$) (i.e., below 1.0) so that they do not reach the stationary phase, during which they have increased autofluorescence. Before fixation, the cells have to go through at least four cell divisions in the freshly inoculated medium. We typically start a culture 1 d before, grow them at a very low density overnight and then re-inoculate them in the morning until they reach an $OD_{600}$ of ~0.5, at which point they are fixed.

### Pretreatment of the slides ● TIMING 2.5 h

**2|** Wash the microscope coverslips by boiling them on the stirring hot plate, in 500 ml of $ddH_2O$ containing 5 ml of 1 N HCl, for 20–30 min while gently stirring to separate the coverslips.

**3|** Rinse the coverslips ten times with $ddH_2O$ and keep them in ~200 ml $ddH_2O$. Use them immediately or autoclave them for long-term storage at 4 °C.

**4|** Lay the coverslips on the chromatography paper; aspirate excess water and air-dry. Coat each one with 200 µl of 1× poly-L-lysine diluted in $ddH_2O$. Incubate the coverslips for 2 min, aspirate and air-dry. Finally, wash the coverslips three times with 250 µl of $ddH_2O$ for 10 min and air-dry.

**5|** Use forceps to place each coverslip into a single well of a six-well tissue culture dish and store the dish at RT.

### Labeling RNA FISH probes ● TIMING Overnight

**6|** Mix 5 µg of unlabeled FISH probe mixture and 1 mg of fluorescent dye in 20 µl of probe labeling buffer (pH 9.0). Vortex thoroughly, touch-spin and incubate the mixture in the dark at RT overnight.

▲ **CRITICAL STEP** Increasing the amount of DNA relative to the dye will greatly decrease the labeling efficiency of the probe. We typically label FISH probes with Cy3, Cy3.5 or Cy5 monoreactive fluorescent dyes; decreasing the pH of the buffer solution will greatly decrease the probe labeling efficiency.

**7|** Purify the probes using the QIAquick nucleotide removal kit according to the manufacturer's instructions.

▲ **CRITICAL STEP** The labeled probe sample is passed through the columns twice to increase the yield of the purified probes and the columns are washed three times to thoroughly remove unbound dye. We find that carryover of the free fluorescent dye into the probe samples is negligible and does not contribute substantially to the fluorescent cellular background.

**8|** Elute the probes with 100 µl of $ddH_2O$.

**9|** Calculate probe labeling efficiency (LE) using Beer-Lambert's law such that $LE = \frac{[dye]}{[probe]N}$ where $N$ is the number of incorporated fluorophore binding sites on a FISH probe. Measure the absorbance with a spectrophotometer (such as a NanoDrop). Measure DNA absorbance at 260 nm, Cy3 absorbance at 552 nm, Cy3.5 absorbance at 581 nm and Cy5 absorbance at 650 nm. The extinction coefficient for DNA can be calculated by using a number of online resources, whereas the extinction coefficient of the fluorescent dye is provided by the manufacturer. For GE Healthcare/Amersham Cy3, Cy3.5 and Cy5 dyes they are as follows: $Cy3_{552}$ = 150,000 $M^{-1}$ $cm^{-1}$, $Cy3.5_{581}$ = 150,000 $M^{-1}$ $cm^{-1}$ and $Cy5_{650}$ = 250,000 $M^{-1}$ $cm^{-1}$. The concentration of the dye is $[dye] = \left[\frac{A_{dye,\tau}}{\varepsilon_{dye,\tau} l}\right]$ and the concentration of the probe is $[probe] = \frac{A_{260} - A([dye]\varepsilon_{dye,260} l)}{\varepsilon_{probe} l}$, where $l$ is the length of the light path in cm, $A$ is absorbance and ε is the extinction coefficient. Extinction coefficients of dyes at 260 nm are 4,930 $M^{-1}$ $cm^{-1}$ ($\varepsilon_{cy3,260}$), 24,000 $M^{-1}$ $cm^{-1}$ ($\varepsilon_{cy3.5,260}$) and 10,000 $M^{-1}$ $cm^{-1}$ ($\varepsilon_{cy5,260}$). A labeling efficiency of 1.0 indicates that all modified sites on the DNA are bound by the dye. Typically, labeling efficiencies above 0.90 are achieved.

**? TROUBLESHOOTING**

### Fixation ● TIMING ~2.5 h

**10|** To fix cells, add 8 ml of 32% (wt/vol) paraformaldehyde to 42 ml of culture from Step 1 and mix it immediately in a 50-ml falcon tube. Incubate the tube at RT for 45 min and invert it every 10 min.

▲ **CRITICAL STEP** Excessive fixation periods will reduce the efficiency of yeast cell wall removal during lyticase treatment.

**! CAUTION** Paraformaldehyde is toxic and should be handled in the fume hood and discarded according to relevant environmental health and safety instructions.

**11|** Wash the cells three times with 10 ml of ice-cold 1× buffer B and centrifuge them at 2,400*g* for 5 min at 4 °C after each wash.

**12|** Resuspend the cells in 1 ml of spheroplasting buffer and 800 U of lyticase.

**13|** Incubate the cells for 10 min at 30 °C, inverting frequently.
▲ **CRITICAL STEP** Duration of lyticase treatment varies with the strain used. Some strains need much longer incubation times; however, for most strains, overdigestion occurs after 10 min and cells will start to lose their morphology. A good way to determine when the cell wall is adequately removed is with a light microscope and bright-field illumination. After digestion, cells will appear opaque.

**14|** Centrifuge the cells for 4 min at 1,300*g* at 4 °C and wash them once in 1 ml of ice-cold 1× buffer B.
▲ **CRITICAL STEP** Do not vortex. Cells are very sensitive to distortion and lysis after lyticase treatment.

**15|** Resuspend the cells in 1 ml of 1× buffer B. Drop ~150 µl on each poly-L-lysine-coated coverslip from Step 5.

**16|** Incubate for 30 min at 4 °C to allow the cells to adhere to coverslips.

**17|** Wash with 5 ml of ice-cold 1× buffer B, aspirate and add 5 ml of 70% ethanol. Seal with Parafilm and store at −20 °C until use.
▲ **CRITICAL STEP** Ethanol (70%, vol/vol) allows long-term storage of the cells for several months. It also perforates the cell membranes and enables the FISH probes to penetrate into the cells during hybridization.
■ **PAUSE POINT** Coverslips have to be incubated at −20 °C for at least 20 min before use and can be stored at this temperature for up to 2 months.

**Hybridization** ● **TIMING ~5.5 h**
**18|** Rehydrate the coverslips in a Coplin jar filled with 2× SSC twice for 5 min at RT.

**19|** Incubate the coverslips in hybridization solution for 15 min at RT.

**20|** Per coverslip, combine 10 ng of total probe mixture from Step 8 and 4 µl of 10 mg $ml^{-1}$ DNA competitor and dry in a Vacufuge.

**21|** Resuspend in 12 µl of solution F.

**22|** Add 12 µl of solution H, mix thoroughly and drop 20 µl into a Petri dish.

**23|** By using forceps, take a coverslip, remove extra hybridization solution and place it face down onto the probe drop. Add a Falcon tube cap full of hybridization solution to humidify the hybridization chamber, cover the Petri dish, seal it with Parafilm and incubate it at 37 °C for 3 h.
▲ **CRITICAL STEP** The hybridization solution containing 40% (wt/vol) formamide is optimized for the FISH probes that are ~50 nt long with a GC content of 50%. Lower formamide concentration will favor nonspecific probe binding, whereas higher formamide concentration will prevent even the specific binding of probes to target mRNA.
■ **PAUSE POINT** Hybridization can be carried out overnight and the rest of the protocol can be continued in the morning.

**24|** Use forceps to place the coverslips back into the Coplin jar; wash them twice in hybridization solution and incubate for 15 min at 37 °C.

**25|** Wash the coverslips with 0.1% (vol/vol) Triton X-100 in 2× SSC at RT, while lightly shaking on the orbital shaker for 15 min.
▲ **CRITICAL STEP** During this and subsequent steps cover the Coplin jar with aluminum foil to prevent bleaching. Prolonged Triton X-100 treatment will excessively perforate the cellular membranes, which will result in loss of cytoplasmic content during subsequent washes.

**26|** Wash twice with 1× SSC for 15 min at RT with light shaking.

**27|** Incubate in a DAPI solution for 10 s.

**28|** Wash with 1× PBS for 5 min.

**29|** Air-dry the coverslips.

**30|** Use forceps to mount the coverslips onto the microscope slides with a mounting medium and allow them to cure.
■ **PAUSE POINT** Certain commercially available mounting media, like ProLong Gold, need to cure for ~24 h at RT before they can be imaged. Water-based mounting media do not need this step and can be imaged immediately after the coverslips are sealed onto the microscope slides with nail polish. If they are stored at −20 °C, the slides can last for months before being imaged.

**Microscopy ● TIMING 1–2 h**
**31|** By using a wide-field epifluorescence microscope (see EQUIPMENT SETUP), optically section the cells with a 200-nm *Z* step, spanning a 4-µm *Z* depth. Exposure times of 700–1,000 ms are typically used to acquire each plane in the Cy3, Cy3.5 and Cy5 channels and ~25 ms exposure times are used to acquire each plane in the DAPI channel.
▲ **CRITICAL STEP** Longer exposure times will rapidly bleach the fluorescent signal. To increase the fluorescence intensity of a single mRNA it is better to increase the number of probes hybridizing to an mRNA rather than to increase the exposure time during imaging.
**? TROUBLESHOOTING**

**Single-molecule detection ● TIMING ~1 d (depends on the proficiency of the user)**
**32|** Maximally project 3D acquired images onto a single *x*,*y* plane (**Fig. 1a**) using an ImageJ 3D Projection Volume Rendering plug-in or any other image-processing algorithm.
▲ **CRITICAL STEP** Detection of fluorescently labeled mRNAs in a 3D image is computationally intense. Instead, a single *x*,*y* image composed of the pixels with the maximal fluorescence intensity in a *z*-stack is analyzed. As a consequence, spots with similar fluorescence intensities positioned in pixels directly above or below each other will maximally project as single spot without the summation of fluorescent signal. Maximal projection, therefore, will lead to undercounting due to overcrowding when a highly expressed or localizing mRNA is analyzed (**Fig. 2a**,**b**). We have determined that in budding yeast undercounting typically begins when 30–40 mRNAs or more are expressed per cell[31]. Before maximal projection and subsequent single-molecule detection, the images can be deconvolved using the Huygens deconvolution algorithm. This algorithm allows the recovery of objects from images that are degraded by blurring and noise. By deconvolving an image, a signal-to-noise ratio of a fluorescently labeled mRNA can increase, which can ease subsequent single-molecule detection.
**? TROUBLESHOOTING**

**33|** Detect single mRNAs using the spot-detection algorithm Localize[12] (**Fig. 1a–c**, see Experimental design). By fitting a 3D Gaussian mask over each fluorescent spot, Localize determines an average integral fluorescence intensity of each mRNA (in arbitrary units; **Fig. 1b**). Plot the distribution of the number of detected spots relative to increasing value of integral fluorescence intensity as a histogram (**Fig. 1c**). In a microscope image, the distribution of fluorescence intensities of detected spots can be approximated by a Gaussian distribution. Thus, the average integral fluorescence brightness of a single spot can be determined if the distribution of integral fluorescence intensities plotted as a histogram is fitted to a Gaussian curve and the peak of the Gaussian curve determined. By using the SigmaPlot data analysis program (or any other data analysis program), fit this distribution (black circles) to a Gaussian curve (red line) to calculate an average intensity of a single FISH-labeled mRNA (**Fig. 1c**).
▲ **CRITICAL STEP** During the single-mRNA detection it is essential to determine appropriate threshold parameters that will allow detection of FISH-labeled mRNAs but will prevent detection of background spots with lower fluorescence intensities. A good way of achieving this threshold is by using multiple FISH probes per target mRNA that are highly efficiently labeled (see Steps 6–9) and that efficiently hybridize to the target mRNA (see Step 34). Typically in budding yeast, at least four probes are used with a labeling efficiency >90%, which strongly amplifies the signal-to-noise ratio and detection sensitivity. Labeled mRNAs are thus readily observed as bright fluorescent spots in a microscope image (**Fig. 1a**).

**34|** Verify that the detected fluorescent spots are indeed single mRNAs. Use Localize to measure an average integral fluorescence intensity of a single FISH probe in order to validate the choice of threshold parameters in Step 33. Individual FISH probes nonspecifically hybridize to the cellular background, however, their fluorescence should be much less than that of

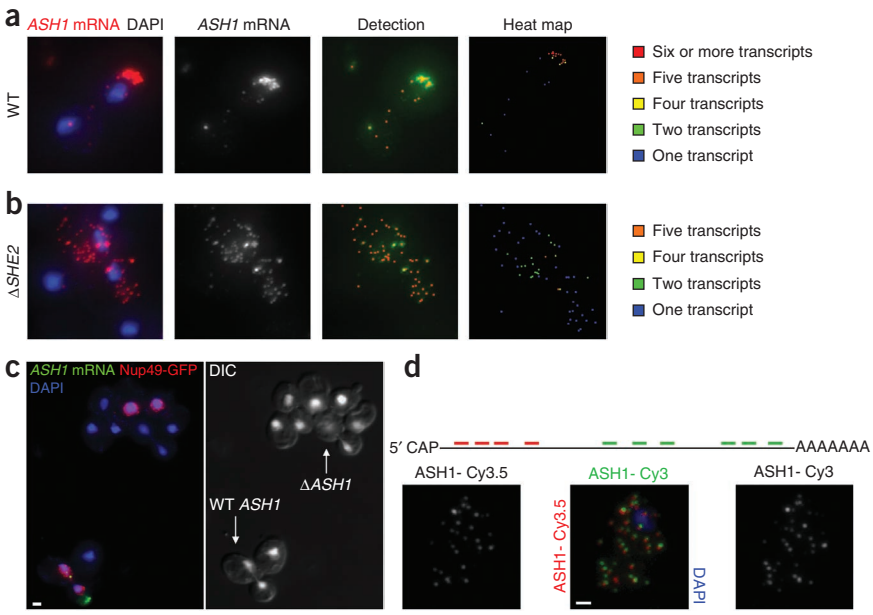


**Figure 2** | Single-molecule FISH controls. (**a**,**b**) Detection of *ASH1* mRNAs (shown in red) in wild-type (WT) cells and Δ*SHE2* cells. DAPI-stained nucleus is shown in blue. Single mRNAs detected with Localize are depicted as red spots. The green background in the 'Detection' image corresponds to the FISH-labeled mRNAs. The images were not deconvolved in Huygens before single-molecule analysis. Deletion of She2p causes a complete delocalization of *ASH1* transcripts[6]. Heat map spatially and quantitatively demonstrates the effect of the *SHE2* deletion on the localization of *ASH1* mRNAs. (**c**) Specific FISH signal can only be detected in the presence of the target mRNA. *ASH1*-expressing cells (green), with the GFP-labeled nuclear pores (Nup49p-GFP; red) were mixed with Δ*ASH1* cells. Nucleus staining with DAPI is shown in blue. Right, the DIC image is overlaid with the DAPI image. *ASH1* expression is cell cycle regulated and reaches peak after cells transition into anaphase phase during mitosis. WT and Δ*ASH1* anaphase cells are indicated with an arrow. (**d**) 5′ and 3′ mRNA ends, labeled with spectrally distinct FISH probes, colocalize. The 5′ end of the *ASH1* mRNA was hybridized with four Cy3.5-conjugated FISH probes and the 3′ end with six cy3-conjugated FISH probes. Narrow band-pass filters, the Chroma filter sets SP102v1 (Cy3) and SP103v1 (Cy3.5) are used to distinguish between the Cy3 and Cy3.5 emission signals. Nucleus staining with DAPI is shown in blue. Scale bars, 1 μm.

a single mRNA. As in Step 33, plot the distribution of the number of detected spots relative to increasing value of integral fluorescence intensity as a histogram. Fit this distribution (black circles) to a Gaussian curve (red line) to calculate an average intensity of a single FISH probe (**Fig. 1d**). For example, in **Figure 1c**,**d**, an average intensity of a single mRNA was 10.5 ± 0.2 photoelectron counts ($R^2$ = 0.85), whereas an average intensity of a single probe hybridizing to the background was 2.6 ± 0.1 photoelectron counts ($R^2$ = 0.99). The fluorescence intensity of a single *ASH1* mRNA was four times greater than that of a single probe indicating that single *ASH1* mRNAs were hybridized by all four probes used in a mixture. Thus, hybridization of probes to a target mRNA was ~100% efficient.

▲ **CRITICAL STEP** Threshold parameters will determine which fluorescent spots will be included in the analysis. If the threshold is set too high, the algorithm will detect only the brightest fluorescent spots (those that can contain multiple mRNAs in the same pixel), which will skew the center of the Gaussian fit toward higher fluorescence intensities. If it is set too low, pixels with higher background fluorescence will also be detected skewing the Gaussian toward lower fluorescence intensities. The average fluorescence intensity of a single mRNA should be equal to the average fluorescence intensity of a single FISH probe multiplied by the number of probes used to label the mRNA as demonstrated in **Figure 1c**,**d**.

**? TROUBLESHOOTING**

**35|** Normalize the fluorescence intensity of each spot by the average fluorescence intensity of a single mRNA to determine the number of mRNAs per detected spot.

**36|** Determine the cellular position of a transcript with sub-pixel resolution through numerical fitting of a FISH-labeled diffraction-limited mRNA. This step is only needed if one is interested in determining the cellular distribution of mRNAs. The approach is described in great detail in ref. 17 and the reader should refer to this paper for a complete protocol. In short, a detection algorithm such as Localize fits a 3D Gaussian mask over a fluorescent spot to determine its integral fluorescent brightness as well as the position of the spot, in which the center of the Gaussian mask represents the location of the spot in *x*,*y* coordinates in a microscope image (**Fig. 1b**). The precision of determining the position of the fluorescent spot depends greatly on the signal-to-noise ratio of the fluorescent spot relative to the cellular background; the higher the fluorescence signal of a spot and/or the lower the background, the better the precision of determining the location of a spot. Thus, the use of multiple, highly efficiently labeled probes hybridizing to one mRNA greatly increases the fluorescence intensity of a FISH-labeled mRNA, and thus the signal-to-noise ratio. Conversely, reducing the fluorescence intensity of the cellular background, either by increasing the number of washes after hybridization of cells with FISH probes or by using cells with minimal autofluorescent background, will also increase the signal-to-noise ratio and thus the precision in determining the location of a fluorescently labeled mRNA. The cellular distribution of spots that contain various numbers of

mRNAs can be visually represented as a heat map (**Fig. 2a**,**b**). To create a heat map, a color is assigned to the spots containing a particular number of mRNAs, where the 'colder' color denotes spots with few mRNAs and a 'hotter' color denotes spots with many mRNAs. A spot-detection algorithm, such as Localize, determines both the fluorescence intensity and cellular position of a spot. The Heat Map can thus be created using Adobe Photoshop by assigning a color to spots with different number of mRNAs.

▲ **CRITICAL STEP** As indicated in Step 32, overcrowding as a result of high expression and/or localization of mRNAs will affect the absolute number of detected single mRNAs.

**37|** Manually segment the cells using the ImageJ plug-in (see EQUIPMENT) to obtain an absolute number of mRNAs per cell (**Fig. 1e**).

## ? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

**TABLE 1** | Troubleshooting table.

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 9 | Poor probe labeling efficiency | Suboptimal probe labeling conditions | Check the pH of the probe labeling buffer. Decrease the probe-versus-dye ratio in the labeling reaction. For Cy3.5, purify the dry mixture using a QIAquick nucleotide removal kit before labeling to remove impurities. This is necessary to achieve high labeling efficiencies with Cy3.5 dye. When an LE of a probe mixture is determined, an average $\varepsilon_{260}$ is assumed in the calculations |
| 31 | Autofluorescence is too high | Old or stressed cells; old coverslips; free fluorescent dye in the probe mixture | Avoid using cells that grew to saturation. Always use fresh cells from the logarithmic phase of their growth curve. Grow cells in complete minimal medium instead of yeast extract peptone dextrose. Prepare new coverslips. Increase the number of washes during purification of labeled probes to remove excess free dye in the probe mixture |
| | The fluorescent signal bleaches | Bleaching due to oxygen radical formation or long exposure time; fluorescent dye is not photostable | Use an appropriate mounting medium, which is an efficient scavenger of oxygen radicals that cause bleaching of the fluorophores. Reduce exposure times. Reduce the number of *Z* steps. Use a more photostable fluorophore. For example, Cy3 is very photostable whereas Cy5 is known to bleach more rapidly |
| | No background FISH probe signal | The yeast cell wall was not digested | The probe did not penetrate into the cells. Check lyticase activity |
| | Too much background FISH probe signal | Nonspecific probe hybridization is too high | Reduce the amount of probe used. Maintain short hybridization periods of ~3 h. Increase washes with the hybridization solution and 2× SSC. Increase formamide concentration in the hybridization solution |
| | Single probes are visible, but not single mRNAs | Bad probe design; poor signal-to-noise ratio; formamide concentration is too high; the gene is not expressed | Probes are designed as sense and not as antisense probes; order new probes. Increase the number of probes used to increase the signal-to-noise ratio. Reduce formamide concentration in the hybridization solution. The gene may not be expressed |
| 32, 34 | The detection algorithm over-or undercounts | Threshold parameters are too high or too low; too many transcripts per cell | Optimize the threshold parameters. Fluorescene intensities of the fluorescent mRNAs/probes change with the exposure time during image acquisition. For each imaged condition, determine the threshold parameters separately. The gene is highly expressed (>30–40 mRNAs/cell, or localized). Spot fluorescence intensity is not normalized by the average intensity of a single mRNA |

● TIMING

Step 1, growing yeast cells for FISH: 48 h
Steps 2–5, pretreatment of the slides: 2.5 h
Steps 6–9, labeling RNA FISH probes: overnight
Steps 10–17, fixation: ~2.5 h
Steps 18–30, hybridization: ~5.5 h
Step 31, microscopy: 1–2 h
Steps 32–37, single-molecule detection: ~1 d (scales according to the user's proficiency)

## ANTICIPATED RESULTS

Quantitative single-molecule FISH is a reliable and highly reproducible technique that has been successfully used in the Singer laboratory for more than a decade. Although the method is typically learned quickly, the main difficulties of the single-molecule FISH protocol are the spot-detection and counting processes: which spot is a single mRNA, how is it distinguishable from background and could there be over- or undercounting of single mRNAs? We find that for most mRNAs, hybridization of probes to their target mRNAs is highly efficient. For mRNAs that do not have a high hybridization efficiency we observe an incomplete binding, usually lacking one probe. Because during hybridization, the 37 °C temperature and high formamide concentration loosen the mRNA-protein interactions and make an mRNA amenable for probe binding, we believe that lower hybridization efficiencies indicate poor probe design. In these cases, a poorly hybridizing probe should be identified and eliminated from the probe mixture.

**Figure 2** shows an example of detection of a localizing *ASH1* mRNA in budding yeast. Expression of *ASH1* is cell cycle–regulated, with the peak of mRNA accumulation after anaphase onset during mitosis. The mRNA is actively localized to the bud tip such that the majority of *ASH1* transcripts are asymmetrically distributed between the mother and the daughter cells (**Fig. 2a**)[6,32]. Several proteins regulate *ASH1* localization, among them She2p. In the absence of She2p, *ASH1* mRNA becomes homogeneously distributed between the mother cell and the bud tip (**Fig. 2b**)[33]. Quantification of *ASH1* mRNA is a good example of troubleshooting of single-molecule FISH. In the wild-type cells, the majority of *ASH1* transcripts are localized in a small volume at a bud tip. We have estimated that during mitosis and after the duplication of the genome, 88 ± 31 *ASH1* mRNAs are produced and transported to the bud tip (data not shown). This overcrowding causes undercounting of *ASH1* mRNAs in maximally projected images (**Fig. 2a**). A better way of determining a total number of *ASH1* mRNAs transcribed is in the absence of She2p, when the transcripts become evenly distributed between the mother and the daughter cell (**Fig. 2b**; note that this is possible, however, if one neglects a possibility that the deletion of She2p might impact *ASH1* transcription or decay rate). When the expression is particularly high, it is important to avoid undercounting due to overcrowding even when an mRNA is homogenously distributed within the cell, as is the case with *ASH1* expressed in the She2p-Δ background. We have estimated that when 65–85 nonlocalized *ASH1* mRNAs are detected in a maximal projection, between 15 and 17% of mRNAs were undetected because of overcrowding (data not shown). In budding yeast, single-molecule FISH on maximally projected images is thus suitable only for the quantification of transcripts with low and moderate abundance.

A particular advantage of single-molecule FISH is the ability to determine the cellular position of a transcript with subpixel resolution through numerical fitting of a FISH-labeled diffraction-limited mRNA[17] (**Fig. 1b**, see Experimental design). Because the precision of determining the position of an mRNA greatly depends on the intensity of an mRNA fluorescence signal relative to the autofluorescent background, the use of multiple efficiently labeled probes per target mRNA, as described in this protocol, is desirable to achieve high location precision. Single-molecule FISH therefore enables the determination of the spatial location of individual transcripts within a cell and relative to any given cellular structure (**Fig. 2a**,**b**)

**Figures 1** and **2** show common controls used to verify single-molecule detection by FISH. The use of a mixture of probes that are efficiently labeled and that further efficiently hybridize to the mRNA is necessary to distinguish between specific and nonspecific FISH signal (**Fig. 1a**–**d**). We take advantage of the nonspecific cellular probe binding and use it to calibrate the fluorescence intensity of a single mRNA by the number of single probes used in a FISH probe mixture (**Fig. 1c**,**d**). This calibration allows the validation of the threshold parameters chosen during the single-mRNA detection. Single probes stick to the poly-L-lysine–coated microscope slide outside of the cell as well; alternatively, when the autofluorescent cellular background is exceptionally high, an average intensity of these single probes can be determined to estimate the fluorescence intensity of a single mRNA.

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