mRNA quantification using single-molecule FISH in *Drosophila* embryos

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Spatial information is critical to the interrogation of developmental and tissue-level regulation of gene expression. However, this information is usually lost when global mRNA levels from tissues are measured using reverse transcriptase PCR, microarray analysis or high-throughput sequencing. By contrast, single-molecule fluorescence *in situ* hybridization (smFISH) preserves the spatial information of the cellular mRNA content with subcellular resolution within tissues. Here we describe an smFISH protocol that allows for the quantification of single mRNAs in *Drosophila* embryos, using commercially available smFISH probes (e.g., short fluorescently labeled DNA oligonucleotides) in combination with wide-field epifluorescence, confocal or instant structured illumination microscopy (iSIM, a super-resolution imaging approach) and a spot-detection algorithm. Fixed *Drosophila* embryos are hybridized in solution with a mixture of smFISH probes, mounted onto coverslips and imaged in 3D. Individual fluorescently labeled mRNAs are then localized within tissues and counted using spot-detection software to generate quantitative, spatially resolved gene expression data sets. With minimum guidance, a graduate student can successfully implement this protocol. The smFISH procedure described here can be completed in 4–5 d.

INTRODUCTION

In situ hybridization allows qualitative mRNA detection in intact tissues and has been a tool of choice for studying the distribution of mRNAs in biological samples for nearly 40 years. However, since the development of smFISH—in combination with a subsequent optimization in 1998 that allows for the counting of single mRNAs[1]—interest in detecting and quantifying all aspects of mRNA biology in individual cells with single-mRNA sensitivity has rapidly increased. This mounting interest was further augmented in 2008 by the development of an smFISH protocol that used a mix of short, singly labeled DNA probes (single-stranded DNA oligos) to detect individual mRNAs in single cells[2], which was accompanied by the subsequent launch of custom-designed, commercially available and affordable Stellaris RNA probes. The year 2015 alone recorded 133 publications that used these commercially available smFISH probes to detect mRNAs[3]; strikingly, nearly half of these studies characterized mRNA expression quantitatively rather than qualitatively. These publications demonstrate that the broader scientific community is interested in not only whether the mRNA is present in the cell but also in how much of it is present and where in the cell it is located. Indeed, several groups have developed algorithms to quantify various aspects of mRNA biology with single-molecule sensitivity in, for instance, *Drosophila*[4–8]. Although use of a spot-detection algorithm for detection and counting of single smFISH-labeled mRNA molecules in *Drosophila* embryos is a critical part of quantitative smFISH, a step-by-step protocol describing this aspect of quantitative smFISH has been absent from the *Drosophila* literature. As the field is rapidly evolving, we describe here an up-to-date protocol for quantitative smFISH—used with cutting-edge microscopy and data analysis software—that will be easy for a broad audience to use.

Overview of the procedure

The smFISH labeling and counting protocol described here is composed of four parts: embryo collection and fixation (Steps 1–9), embryo hybridization with smFISH probes (Steps 10–26), imaging (Step 27) and single-mRNA detection and counting (Steps 28–42).

Drosophila embryos are collected, dechorionated and fixed with paraformaldehyde and paraformaldehyde-saturated heptane. Fixative is then removed and methanol is added, which helps to remove the vitelline membrane surrounding the embryo and allows penetration of smFISH probes into the sample. After the vitelline membrane is removed, embryos are washed with methanol and stored overnight at 4 °C in methanol.

The next day, the embryos are rehydrated and postfixed with paraformaldehyde, followed by permeabilization with detergent. The embryos are then treated with Proteinase K to allow the smFISH probe to penetrate deeper into the embryo volume; this is followed by an additional fixation with paraformaldehyde. During a prehybridization step, embryos are incubated in deionized formamide, which equilibrates the sample for a subsequent hybridization with smFISH probes. Embryos are then resuspended in the hybridization mix containing smFISH probes and left to incubate overnight at 37°C. The next day, the embryos are mounted onto coverslips and imaged using either a wide-field epifluorescence, confocal or iSIM instrument (a super-resolution imaging approach; see the 'Equipment' section). Afterward, 3D images are deconvolved and single mRNAs are detected and counted using a freely available spot-detection algorithm, Airlocalize, which was developed in the MATLAB programming language (MathWorks)[9] (see the 'Equipment' section).

Development of the protocol

Our groups have previously published articles on the biological applications of mRNA labeling using commercially available smFISH probes in *Drosophila*[5], the development of the iSIM

super-resolution system[10,11], and the application of Airlocalize, the algorithm that enables detection and counting of smFISH-hybridized mRNAs[5,9,12]. The current protocol covers all steps in sufficient detail to guide nonspecialist users, and has an emphasis on single-molecule counting, arguably the most complex step.

Our fluorescence staining protocol for mRNAs in *Drosophila* embryos is robust, reproducible and easy to implement. The protocol detailing embryo fixation and collection, followed by embryo hybridization with smFISH probes, is a modification of protocols by Lecuyer *et al.*[13], Lionnet *et al.*[9], Femino *et al.*[1] and Raj *et al.*[2]. To count individual mRNAs in *Drosophila* embryos, we use multiple short, singly labeled commercially available smFISH probes[2,5], rather than the one long and heavily fluorescently labeled RNA probe traditionally used in whole-mount *in situ* studies[13]. In a whole-mount *in situ* approach, a single antisense RNA probe is transcribed from a DNA template *in vitro* in the presence of digoxigenin-UTP (DIG-UTP). The resulting FISH probe is subsequently hybridized to the target mRNA. DIG-modified FISH probes are not readily visualized *per se*. Rather, DIG is detected with enzyme-coupled anti-DIG antibodies. These enzymes, such as alkaline phosphatase, react with chromogenic substrates to reveal the spatial distribution of target mRNAs[14]. The chromogenic dyes produced by alkaline phosphatase, however, rapidly diffuse away from the target mRNA, thereby reducing the spatial resolution of the technique[13–15]. This shortcoming was mitigated by the introduction of horseradish-peroxidase-mediated deposition of tyramide radicals labeled with fluorescent dyes (such as Cyanine-3), which covalently react with the proteins at the site of horseradish peroxidase activity. The fluorescent signal becomes localized and the spatial resolution of the technique increases[13,15]. Whole-mount *in situ* approaches have been widely adopted to visualize mRNAs, particularly in the fruit fly. In *Drosophila*, initial, critical steps of the fly development rely entirely on post-transcriptional regulation. Strikingly, almost 1,700 maternally deposited mRNAs are localized within a developing embryo[16]. Because of its strong staining potential (every 20th–25th nucleotide in the RNA FISH probe is a DIG-UTP whose detection is further enzymatically amplified), whole-mount *in situ* studies have become an invaluable tool for studying how post-transcriptional gene expression such as mRNA localization drives early *Drosophila* development. However, all FISH probes also non-specifically bind to the cellular background[17–19] (see below and also the 'smFISH probes' section in the 'Experimental design'). Without the ability to distinguish between a *bona fide* hybridization of the probe to the target mRNA versus the cellular background, whole-mount *in situ* studies can provide only qualitative rather than quantitative information. Our protocol uses multiple short, singly labeled smFISH probes[2,5] that bind to different positions on the target mRNA to fluorescently label mRNAs *in situ*. As a result, the right combination of smFISH probes can be found only on the target mRNA and not on other mRNAs or the cellular background. Thus, target mRNAs will always appear as bright fluorescent spots against a uniform fluorescent background. This approach allows us to readily distinguish between specific binding to the target mRNA and nonspecific sticking of the probe to the cellular background (see below). The commercial availability of the probes and the relative ease of their synthesis make it possible to adapt our protocol to quantify various aspects of mRNA biology in *Drosophila* embryos with subcellular resolution[5–8,20,21].

Stained *Drosophila* embryos are imaged in 3D to allow quantification of uniformly distributed and localized mRNAs in a volume[5,9]. Our smFISH staining protocol is compatible with a range of imaging modalities, including wide-field and confocal microscopy, as well as structured illumination microscopy (SIM), a super-resolution method. SIM offers a more modest two-fold resolution improvement relative to other super-resolution microscopy techniques (e.g., localization microscopy or stimulated emission depletion microscopy) but is much faster, requires much less illumination (causing less photobleaching) and does not require special fluorophores or sample preparation. These advantages make SIM particularly well suited for our protocol. Here we describe the application of a variation of SIM called iSIM[10,11]. Unlike conventional SIM, iSIM offers very rapid imaging (no loss of speed relative to wide-field epifluorescence microscopy, achieved by performing resolution enhancement optically in the microscope hardware) and enables superior imaging in thick samples because of better background rejection (due to pinholes that reject out-of-focus light). iSIM therefore provides superior detection sensitivity, as well as superior localization precision during fluorescent mRNA detection. When coupled with smFISH, iSIM allows a highly detailed quantification of the spatial distribution of transcripts within a complex protein environment such as an mRNA-bound germ granule[5], otherwise unattainable with wide-field epifluorescence or confocal microscopy.

3D images are analyzed with a MATLAB-based custom spot-detection algorithm, Airlocalize[5,9], to detect and count single, smFISH-labeled mRNAs. Individual, fluorescently labeled transcripts can be detected with subpixel resolution in 2D images, as well as in 3D stacks (available upon request). A local affine baseline subtraction step computes and subtracts the background fluorescence pattern surrounding each mRNA spot. As a result, the intensity measurements are robust even in the presence of high fluorescence background, as is often encountered in tissue. By providing a comprehensive guide to single-molecule detection and counting, Airlocalize can be implemented to quantify the cellular mRNA content in any context[5,9,12].

Application of the method

Although we showcase the *Drosophila* embryo as an example in this protocol, the mRNA staining with smFISH probes, imaging and image analysis techniques can be implemented to quantify cellular mRNAs in a variety of tissues and organisms. Unlike the long, *in vitro*-transcribed RNA probes used in whole-mount *in situ* studies, the smFISH probes used in this protocol are short and thus easily penetrate *Drosophila* oocytes, regardless of their developmental age. After oocytes are extracted from the female fruit flies, the smFISH staining protocol outlined here can be easily implemented to fluorescently label mRNAs expressed during all oogenic stages (**Box 1**). smFISH has been implemented to characterize mRNA transcription[2,4,7,22–31], splicing[32–34], nuclear export[34,35], localization[5,6,8,17,21,29,36–41] and decay[35,42–44] in single cells or in developing organisms using a variety of imaging approaches. It has also been used to quantify expression and localization of long noncoding mRNAs[45,46], to visualize genomic loci in fixed cells[47] and to identify sites of active mRNA translation[12]. The mRNA staining protocol described here is compatible with a range of imaging modalities, including conventional wide-field epifluorescence and confocal microscopy, as well as SIM[5,9,12]. Our

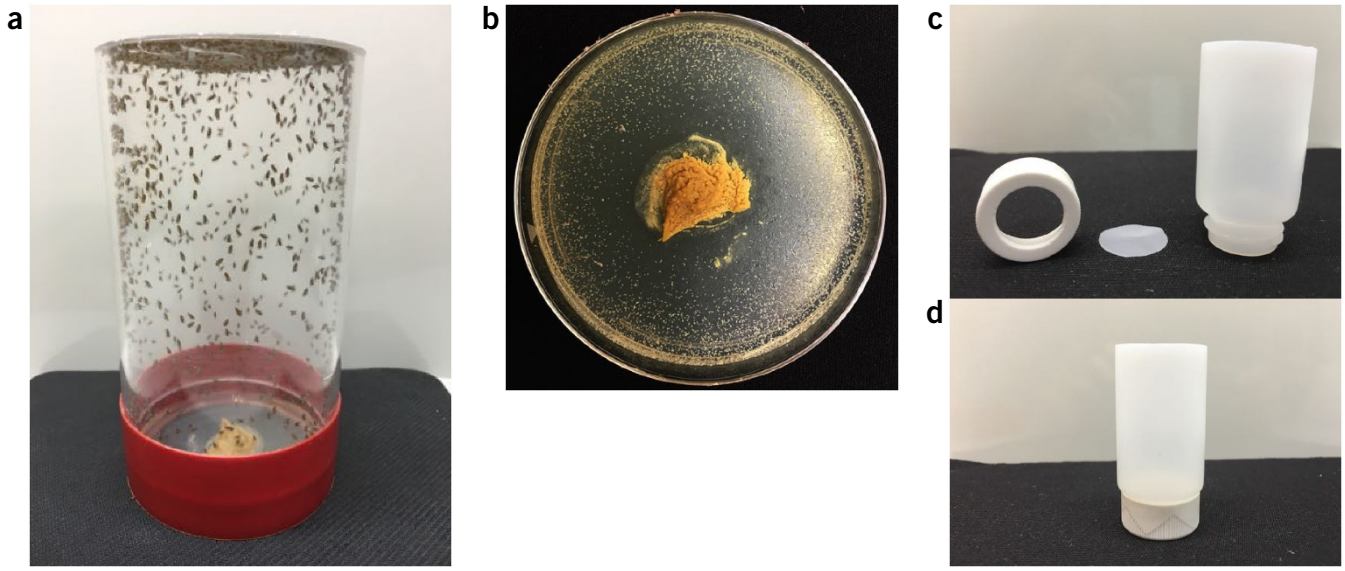

Figure 1 | Collection and fixing of fly embryos for smFISH. (**a**) Egg-collection cage housing fruit flies, with an apple juice plate containing yeast paste. (**b**) Apple juice plate with yeast paste and laid embryos. (**c**,**d**) The egg-collection basket before (**c**) and after (**d**) assembly.

image analysis protocol can be used in diverse cell types, tissues and organisms[5,9,12] to quantify uniformly distributed[5,9,12], localized[5,9] and translating mRNAs[12] (Anticipated Results). By using smFISH probes hybridizing to introns of nascent mRNAs, our smFISH protocol also enables quantification of transcriptional dynamics by direct visualization of active transcription sites within individual nuclei throughout *Drosophila* development (Anticipated Results).

The analysis protocol relies on our freely available spot-detection algorithm (Airlocalize), which localizes individual, fluorescently labeled transcripts with a precision greatly superior to the diffraction limit in 2D or 3D data sets. With a built-in 'Batch mode' option, Airlocalize makes it possible to process a large series of images in sequence, automating the analysis process. The algorithm also allows the analysis of 3D regions of interest (ROIs) of a known volume; this speeds up the analysis when the signal is restricted to specific regions of large data sets, or when one uses a representative region as a proxy for the concentration of homogeneously distributed mRNAs across the entire embryo[5]. The absolute number of localized mRNAs clustered in a diffraction-limited spot (e.g., germ-granule-localized mRNAs[5]) can also be determined by measuring the cumulative fluorescence intensity of an individual mRNA cluster and normalizing it by the fluorescence intensity value of a single mRNA. Concurrently with the quantification of the absolute number of transcripts, Airlocalize also determines the spatial position of clusters with subpixel resolution. Specifically for mRNA clusters localized in *Drosophila* germ granules and imaged with iSIM, the precision with which we are able to determine the position of smFISH-labeled mRNA clusters in granules ranges between 9.1±2.9 and 14.7±1.6 nm[5]. As single-mRNA counting techniques are being increasingly used to study the mechanisms of gene expression in unperturbed cells and tissues, this approachable user interface, combined with the potential to scale the analysis up to larger data sets, constitutes a unique resource for the smFISH community.

Limitations

When quantifying uniformly dispersed, unlocalized mRNAs, our quantitative smFISH performs exceptionally well, unless the abundance of transcripts exceeds the concentration at which

Box 1 | Preparation of late-stage *Drosophila* oocytes for staining with smFISH probes ● TIMING up to 1 h

To prepare oocytes for smFISH, the ovaries of well-fed, 3- to ~4-d-old female flies are dissected under a dissection microscope on a Petri dish and in cold 1× PBS. After several ovaries are collected, they are immediately resuspended in 1 ml of fixative containing 4% (vol/vol) paraformaldehyde and 1× PBS dissolved in ddH_2O, and are incubated for 20 min at RT. Unlike for embryos, saturated heptane is not needed to fix oocytes. Ovaries are then washed three to five times in 1× PBS, each time for 5 min at RT. Ovaries can then be further dissected to release individual ovarioles or oocytes from the ovary. Before smFISH can precede, ovaries, ovarioles or oocytes must be resuspended in 100% (vol/vol) methanol and incubated overnight (or longer) to allow smFISH probe penetration into the oocytes[6,8]. Afterward, the smFISH staining continues as described for embryos in this protocol, starting with Step 11. However, oocytes are susceptible to proteinase K overdigestion and can quickly lose their morphology during treatment. It is preferable, therefore, that the duration and potency of the proteinase K treatment for oocytes is reduced or that the treatment is omitted during smFISH staining (omitting Steps 16–20) (**Fig. 5f**,**g**).

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the individual transcripts are no longer discernible as diffraction-limited spots (Anticipated Results). An example of such a high-abundant mRNA in the *Drosophila* embryo is *cycB*. Using wide-field epifluorescence, confocal or SIM, *cycB* transcripts are not discernible as single molecules, and the somatic regions of young wild-type (WT) embryos stain with uniform yet intense fluorescence[5]. We have determined experimentally that in WT embryos and within the resolution of iSIM, quantification of mRNA levels with smFISH meets the limit when the concentration of the expressed mRNA target reaches ~4 nM (~17 million copies per WT embryo)[5]. Above this concentration, the algorithm begins undercounting the number of mRNA molecules expressed in the sample. In addition, smFISH is not a whole-transcriptome approach. Rather, the number of mRNAs that can be simultaneously imaged is limited by the number of spectrally distinct dyes that covalently label the probes, as well as by the number of filters, detectors and laser lines accommodated by the microscope (typically up to 3). This limit can be extended to characterize up to tens of distinct mRNAs simultaneously by using a defined color combination of spectrally distinct smFISH probes hybridizing to specific target mRNAs (spectral barcoding using smFISH or sequential hybridization)[48–51]. Recently, a highly multiplexed smFISH imaging method called multiplexed error-robust fluorescence *in situ* hybridization was developed to enable simultaneous visualization, counting and cellular localization of 140 different mRNAs[29]. This is a true high-throughput approach that will find numerous applications in cultured cells, as well as in tissues.

As with any resolution-enhancing implementation of SIM, achieving the full resolution gain (typically twofold better than diffraction-limited microscopy) is dependent on appropriate deconvolution. Such deconvolution relies critically on the nature of the algorithm and point-spread function (PSF, e.g., experimental or theoretical). We have obtained good results with an assumed Gaussian PSF and the Richardson–Lucy deconvolution algorithm[52,53] (as well as with the commercially supported Huygens software), as these choices describe the iSIM PSF sufficiently. However, we note that refractive index mismatch, mounting media and the details of the microscope directly influence the PSF—and thus we urge the reader to verify that the assumed PSF indeed matches the experimentally measured PSF before attempting deconvolution. Suboptimal deconvolution can lead to image artifacts and loss of the spatial resolution/localization precision of this method.

Finally, we note that optical aberrations and the working distance of the objective lens limit the effective imaging depth when using iSIM for this application. We recommend taking care to match the refractive index of the immersion oil, the numerical aperture (NA) of the objective, the refractive index of the mounting medium and the coverslip thickness (no. 1.5) to limit spherical aberration, which would otherwise lower the effective signal-to-noise ratio and spatial resolution. When using a high-index mounting medium and a high-NA oil-immersion lens, the working distance of the objective lens also limits the depth of smFISH—we conducted all experiments up to 40 µm from the coverslip when using a 1.45 NA oil-immersion lens.

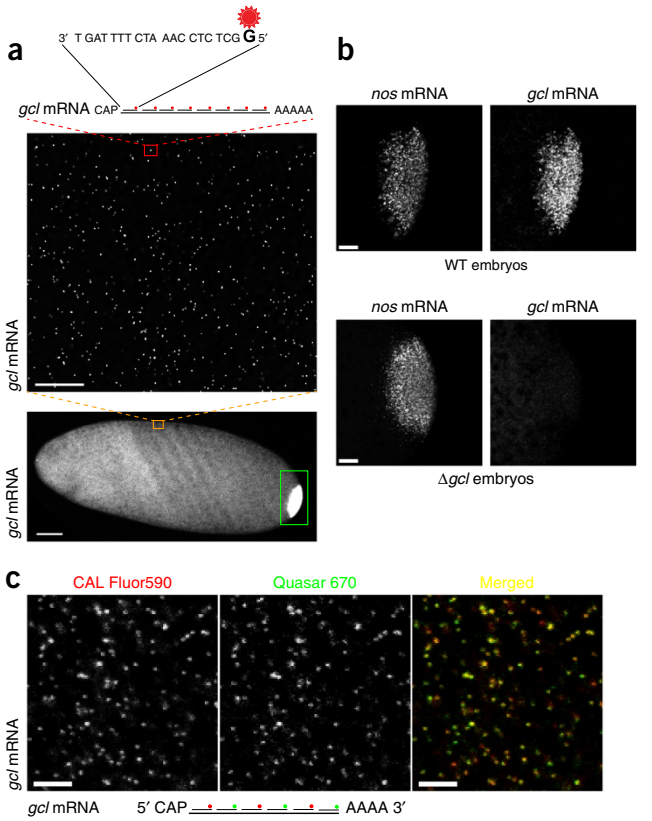

Figure 2 | Detection of single, smFISH-hybridized mRNAs in *Drosophila* embryos. (**a**, top) Schematic of a *germ-cell-less* (*gcl*) mRNA hybridized with a mix of fluorescently labeled, commercially available smFISH probes. (center) *gcl* mRNA, hybridized with 32 smFISH probes; it appears as a bright fluorescent spot (red box) against a uniform autofluorescent embryo background. (Bottom) Uniformly dispersed, single *gcl* mRNAs located ventrally (orange box) in the *Drosophila* embryo. Nearly 3% of all *gcl* mRNA localizes to the embryo's posterior[5] (green box). (**b**) Specificity of smFISH probes to their target RNA; in the absence of *gcl* mRNA expression (in embryos laid by mothers mutated for *gcl* gene expression (*Δgcl* embryos)), the *gcl* smFISH signal cannot be detected, whereas the fluorescence of a control *nanos* (*nos*) mRNA, also enriched at the posterior pole, remains unaffected. (**c**) *gcl* mRNA hybridized with interchanging spectrally distinct CAL Fluor590 (left) and Quasar 670 (center) probes. The right panel shows an overlay of the two fluorescent signals (merged). Scale bars, 5 µm (**a** (center),**c**); 10 µm (**b**); 50 µm (**a** (bottom)). Images in **a** were acquired with a wide-field epifluorescence microscope; those in **b** and **c** were acquired with a laser scanning confocal microscope.

Comparison with alternative methods

An alternative to single mRNA detection using the smFISH probes described here is the use of molecular beacons. These are fluorescently labeled probes that fluoresce only upon hybridization to their target mRNA[54,55]. Molecular beacons are typically short and single-stranded DNA sequences that contain a stem–loop structure. The loop portion of the molecular beacon contains a sequence perfectly complementary to a target mRNA. The stem, which is only present when the molecular beacon is not bound to its target mRNA, is formed upon annealing of complementary sequences in the probe flanking the loop. A fluorophore and a quencher are covalently coupled to each end of the molecular beacon. In the absence of hybridization, the formation of the stem–loop brings the fluorophore and the quencher into close proximity, thereby quenching the fluorescence of the fluorophore. This is a particular advantage of this approach, as it eliminates fluorescence originating from nonspecific probe binding and thereby increases the accuracy of detecting *bona fide* mRNA

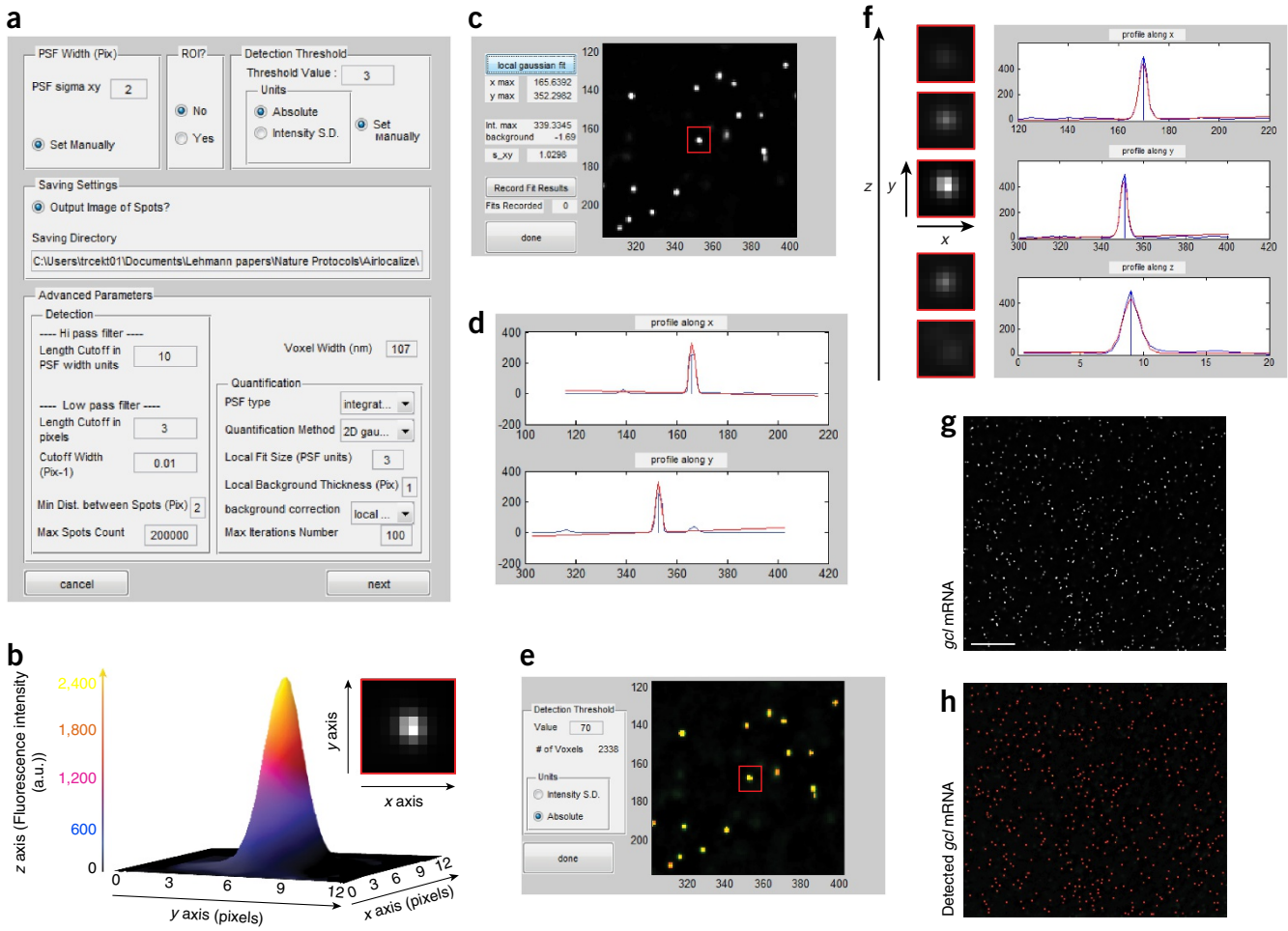

Figure 3 | Workflow of the single mRNA molecule detection using Airlocalize. (**a**) Definition of the microscope parameters for single mRNA detection. (**b**) A heat map demonstrating the spatial distribution of the fluorescence intensity for a single smFISH-labeled *gcl* mRNA, reported in arbitrary units (a.u.). The inset demonstrates a single-plane close-up of a *gcl* mRNA hybridized with 32 smFISH probes acquired on a wide-field epifluorescence microscope. (**c**,**d**) An example of a single fluorescently labeled mRNA (**c**, red box) and its intensity profile (**d**). Blue line in **d** indicates the profile of a fluorescent mRNA along the *x* and *y* axis; red line is the Gaussian fit to the PSF. (**e**) Green spots are smFISH-labeled *gcl* mRNAs. Red spots are *gcl* mRNAs that have a fluorescence intensity greater than the specified detection threshold intensity. Yellow indicates colocalization between smFISH-labeled *gcl* and thresholded *gcl* fluorescent signal. (**f**) Blue lines in profiles indicate the intensity of a fluorescent mRNA along the *x*, *y* and *z* axes, and red lines are the Gaussian fits to the 3D profiles. The five images in **f** are x,y images of a single smFISH-stained *gcl* mRNA from **c** (red box) imaged at different positions. (**g**,**h**) Images showing smFISH-labeled *gcl* mRNAs found in the middle of the embryo (**Fig. 2b**) before (**g**) and after (**h**) spot detection using Airlocalize. In **h**, detected single *gcl* mRNAs are marked as red spots. Scale bar, 5 µm.

targets. When injected into live *Drosophila* oocytes, molecular beacons also enable quantification of mRNAs in live tissue[55,56].

As smFISH is carried out in fixed tissue, it typically provides only a snapshot of an mRNA life cycle. To detect individual mRNAs with a detailed temporal and spatial resolution in living tissue, one can genetically tag mRNAs such that they will bind fluorescent proteins to allow single-molecule detection. Currently, two such systems are most commonly used: the MS2 and the PP7 systems. They are based on the MS2- and PP7-bacteriophage-derived nucleic sequences, respectively[57,58]. These sequences are repeated up to 24 times and are typically inserted into the 3′ untranslated region (UTR) of a gene. Once the mRNA is transcribed, these sequences form stem–loops that enable binding of the MS2 or PP7 bacteriophage capsid proteins, tagged with a fluorescent protein such as GFP. Because a single transcript is tagged with multiple stem–loops, which will each bind a fluorescently tagged capsid protein, the mature mRNA is strongly fluorescently labeled. This multiplication of fluorescent signal on a single mRNA enables single mRNA detection in single living cells, as well as in live *Drosophila* tissue[8,12,58–64]. Development of additional tags such as U1A and λ_N makes it possible to visualize multiple mRNAs in live tissue concurrently[12,57,58,65]. For some mRNAs, genetic tagging of the 3′ UTR of the gene with 24 MS2 or PP7 repeats seems to not affect the life cycle of the mRNA[9,58], whereas for others, the insertion of repeats affects mRNA stability of the target mRNA[66]. Thus, before using MS2- or PP7-like strategies to visualize mRNA, care must be taken to ascertain whether this genetic tagging affects the mRNA's life cycle.

Experimental design

smFISH probes. This protocol uses commercially available Stellaris FISH probes to detect individual mRNA molecules in *Drosophila* embryos. It follows a strategy introduced by Raj *et al.*[2] that was later commercialized by Biosearch Technologies. In short,

a typical smFISH probe is designed as a 20-nucleotide-long DNA primer with a GC composition of ~50% (vol/vol) such that the minimal distance among neighboring probes is two nucleotides. Each probe is covalently conjugated to a single fluorophore via the 5′-most nucleotide and HPLC-purified to remove free dye and unconjugated probes. This purification step is important because it ensures that the uncoupled dye does not contribute to the autofluorescent background during hybridization (non-specific sticking of unconjugated dye in the sample). Unlabeled smFISH probes (those that did not become covalently coupled to a fluorescent dye and are included in the hybridization) will also bind to the target mRNA but will not increase the fluorescence intensity of the target mRNA. mRNAs hybridized with coupled and uncoupled probes will be dimmer and harder to distinguish from the cellular background. In addition, unlabeled smFISH probes will also increase the heterogeneity in the fluorescence intensity among individual mRNA, making the process of detecting single mRNAs less accurate. To improve the specificity of probe binding, stringent masking levels can be implemented to design organism-specific probes and to avoid problematic RNA sequences (https://www.biosearchtech.com/stellarisdesigner). Hybridization of target mRNAs with smFISH probes is carried out in the presence of 10% (vol/vol) formamide and 2× saline-sodium citrate (SSC) buffer at 37 °C, conditions that are optimized to allow optimal hybridization of a 20-nucleotide-long probe with a 50% (vol/vol) GC composition. Changes in the length and GC composition of the probe, as well as changes in the composition of the hybridization mix and in the hybridization temperature, can affect the efficiency of smFISH probe binding to the target mRNA (see the 'Parameters affecting single-molecule detection' section below and the Troubleshooting section where 'No or low smFISH design' is discussed). smFISH probes are purchased as a mix containing up to 48 individual smFISH probes targeting different positions along the target mRNA for each probe (**Fig. 2a**). We most often use probes conjugated to CAL Fluor590, Quasar 570 or Quasar 670. smFISH probes in each probe mix designed to hybridize to the same target mRNA are combined in an equimolar ratio. Applying a mix of smFISH probes that anneal to different positions on the target mRNA strongly improves the signal-to-noise (S/N) ratio and therefore increases detection sensitivity during single-molecule counting. Typically, more probes per target mRNA are desirable for detecting single mRNAs in the embryo; however, one can reliably detect individual transcripts with <48 probes. We have successfully quantified single polar granule component (*pgc*) mRNAs hybridized with 17 smFISH probes conjugated in the laboratory with Alexa488 dye and imaged with iSIM[5]. Similarly, germ-cell-less (*gcl*) mRNAs hybridized with 32 Quasar 670-labeled smFISH probes, imaged using a wide-field microscope, followed by deconvolution, produced adequate signals to reliably quantify the absolute number of this mRNA in the early embryo (**Fig. 4b,c**).

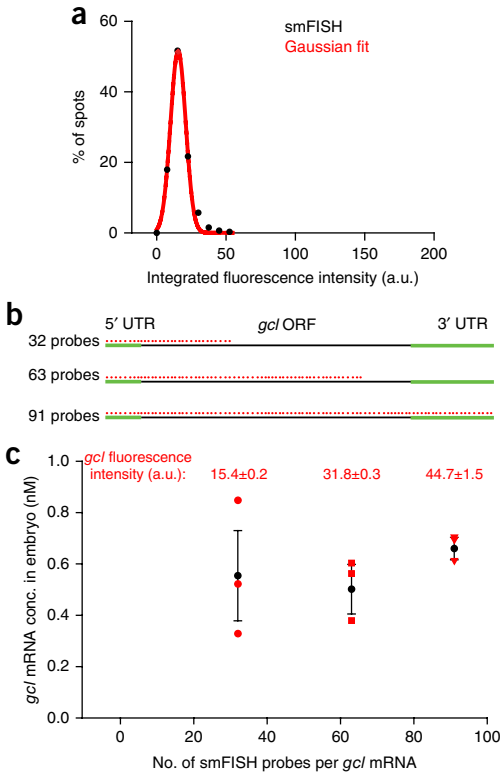

Figure 4 | Determining the fluorescence intensity of a single mRNA molecule. (**a**) The integrated fluorescence intensities of all detected spots from **Figure 3h** are plotted as a histogram (black circles) and fitted to a Gaussian curve (red line). The peak of the Gaussian fit determines the average integrated fluorescence intensity of a single *gcl* mRNA, here hybridized with 32 singly labeled Quasar 670 probes, which, with given imaging and microscope parameters, was 15.4 ± 0.2 a.u. (R^2 = 0.99). (**b**,**c**) *gcl* was labeled with 32 (*gcl* first third (smFISH probes annealing to the first one third of *gcl* mRNA) probe mix), 63 (*gcl* first third and second third smFISH probe mix) or 91 (*gcl* first third, second third and third smFISH probe mix) Quasar 670 smFISH probes (**b**). (**c**) An average integrated fluorescence intensity of a single *gcl* mRNA determined ventrally in the early embryo for each probe mix, as described above, and the concentration of *gcl* mRNA detected for each smFISH probe mix. For each probe mix, an average of three embryos ± s.e.m. is shown. Red circles, squares and triangles represent individual measurements performed on embryos stained with 32, 63 and 91 *gcl* smFISH probes, respectively.

Instant structured illumination microscopy. As mRNAs hybridized with smFISH probes appear as diffraction-limited spots, the ability to resolve features at or below the diffraction limit ('super-resolution') is helpful when attempting to count spots, particularly for spots in close (<250 nm) proximity. Among the plethora of available super-resolution microscopy techniques, linear SIM[6,7] stands out because of its high speed, compatibility with standard fluorescent probes and relatively low photon dose (10^2–10^6 lower than other methods, resulting in far less photobleaching). In SIM, higher spatial resolution (usually twofold improvement over the diffraction limit) is obtained via multiplication of excitation and emission PSF. In practice, this is achieved by exciting the sample with a series of sharp illumination patterns, recording one or more diffraction-limited images and mathematically processing these images. In this protocol, we use a variant of SIM, iSIM[10]. iSIM enables much faster imaging (by a factor of 10–100) than conventional SIM because only a single image is recorded and most of the requisite image processing occurs during image formation (so less digital postprocessing is needed). In addition, and importantly for *Drosophila* samples, iSIM uses a pinhole array to block out-of-focus fluorescence, thus improving background rejection and the signal-to-noise ratio relative to conventional SIM in these thick, densely labeled samples.

Spot-detection algorithm Airlocalize. mRNAs hybridized with smFISH probes appear as bright fluorescent spots that are either uniformly dispersed throughout the embryo or localized to a subembryonic region. To automate the spot detection, an algorithm is used that is capable of detecting individual spots and measuring the number, positions and intensities of these fluorescently labeled mRNAs in a 2D or 3D image. We use an algorithm called Airlocalize, which was developed in the MATLAB programming language (MathWorks)[9]. An image of a fluorescently labeled mRNA corresponds to a diffraction-limited image of a subresolution object (an object with spatial extent less than the resolution of the light microscope). Thus, the size of an mRNA labeled with smFISH probes is determined by the optical properties of the microscope (known as the PSF) and not the physical size of the mRNA molecule. The spot-detection algorithm uses a 3D Gaussian kernel to find the center and intensity of each spot (the kernel is calculated based on the average PSF shape)[9]. The robustness of spot identification and its localization against a high autofluorescent background (for example, created by neighboring fluorescent particles in a crowded environment) is enhanced by an affine local background subtraction before applying the Gaussian kernel. Detection and quantification of smFISH-labeled mRNAs proceeds through the following steps (**Fig. 3**): (i) a spatial band-pass filter is implemented to identify candidate spots for further analysis, (ii) the local background surrounding the fluorescent spots is subtracted from the integral fluorescence intensity of a spot to remove any residual offset or unevenness in the image, (iii) 2D (for 2D images) or 3D (for 3D images) Gaussian mask fitting of each fluorescent spot is implemented to find the center and the intensity of each spot and (iv) spots are culled based on duplication and/or an intensity threshold. Spot intensities and positions are written to a data file for further analysis (**Figs. 3** and **4**).

Parameters affecting single-molecule detection. Typically, fluorescence intensity is the critical parameter for differentiating specific signal from background originating from nonspecific probe binding, and thus care must be taken in any algorithm to ensure the best possible recovery of the spot intensity from the image. A number of controls described in the 'Experimental design' and 'Anticipated Results' sections can be used to differentiate between specific and nonspecific fluorescence signal during the spot detection step. Often, mRNAs are grouped to form a cluster in a smaller volume such that individual smFISH-labeled mRNAs within a cluster cannot be individually resolved[5]. To determine the absolute number of mRNAs grouped within a smaller volume (clustered mRNAs), the integrated fluorescence intensity of a spot is first determined, and is then calibrated by the fluorescence intensity value of a single mRNA located outside of the cluster (**Fig. 5d**). When mRNAs labeled with smFISH probes are particularly heavily clustered in a small volume (as is the case with germ granule localized mRNAs[5]), the abundance of fluorescent signal emanating from the cluster can saturate the dynamic range of the camera or the detector of the microscope. For example, a charge-coupled device (CCD) camera mounted on a wide-field epifluorescence microscope typically does not provide enough dynamic range to concurrently image single and heavily clustered transcripts such as *nanos* (*nos*) mRNA. Because *nos* forms clusters that can contain more than 45 mRNAs packed into a volume comparable to the resolution limit of the wide-field epifluorescence microscope, the multitude of smFISH probes within a cluster produces enough signal to saturate the camera. Such images are not quantifiable. This problem can be mitigated by reducing the number of probes in the probe mix while making sure that the single mRNAs are labeled with enough probes that they are still observable as single mRNAs (see above, 'smFISH probes' in the 'Experimental design' section). Alternatively, a detector or a camera with increased dynamic range can be selected to better match the sample. For example, the ~30,000:1 dynamic range of the scientific complementary-metal-oxide-semiconductor (cMOS) camera mounted on our iSIM (see the 'Microscope' section in the 'Equipment Setup' section) reliably captures the full fluorescence intensity profile of a single and of clustered *nos* transcripts without signal saturation and without the need to reduce the number of smFISH probes during hybridization.

In addition, there are two main sources of background that deteriorate image quality and reduce the S/N ratio and single-molecule sensitivity in the fly embryo. First is a very high autofluorescence originating from the embryo's yolk, particularly in the green and red channels. Second is the fluorescence originating from nonspecific smFISH probe hybridization to cellular background. To overcome autofluorescent background and to achieve single mRNA detection sensitivity, our protocol uses multiple short, singly labeled smFISH probes to fluorescently label mRNAs *in situ* (**Fig. 2a** (top)) rather than one long *in vitro*-transcribed RNA probe labeled with multiple fluorescent dyes to enhance detection capability traditionally used in whole-mount *in situ* studies to detect transcripts in *Drosophila*[13]. This probe iteration strongly amplifies the fluorescent signal of labeled mRNAs, which afterward appear as bright fluorescent spots against a uniform autofluorescent background (**Fig. 2a** (center)). We find that reducing the autofluorescent signal is particularly challenging, and efforts to reduce it result only in minor changes in its intensity. When the autofluorescent signal is particularly high, multiple smFISH probe mixes can be designed to hybridize to 5′ and 3′ untranslated regions (UTRs), as well as the open reading frame(ORF), to further augment the fluorescence intensity of the smFISH-hybridized mRNA relative to the autofluorescent background. smFISH probes also nonspecifically hybridize to cellular background[17,18]. The length and the GC content of the smFISH probes we use ('Reagent Setup') are designed such that all the probes in a probe mix optimally hybridize to the target mRNA at 37 °C in the presence of 10% (vol/vol) formamide and 2× SSC. Care must be taken to prepare a prehybridization solution with 10% (vol/vol) formamide and 2× SSC and to make sure that the hybridization of the sample with the probe mix proceeds at 37 °C. Lower formamide concentration and lower hybridization temperature will encourage nonspecific probe binding and increase background fluorescence. A higher formamide concentration and a higher hybridization temperature will prevent hybridization of the probe to the target mRNA. Exposed to air, deionized formamide also quickly hydrolytically breaks down to formic acid and ammonium formate, and acidifies. Acidified formamide quenches (inhibits) the fluorescence of certain organic dyes such as Quasar 670 during hybridization. To prevent acidification, formamide can be divided into aliquots and stored at −80 °C until use. Monitor the pH of the stored deionized formamide using a pH strip. A pH between 7.5 and 8 indicates good-quality deionized formamide.

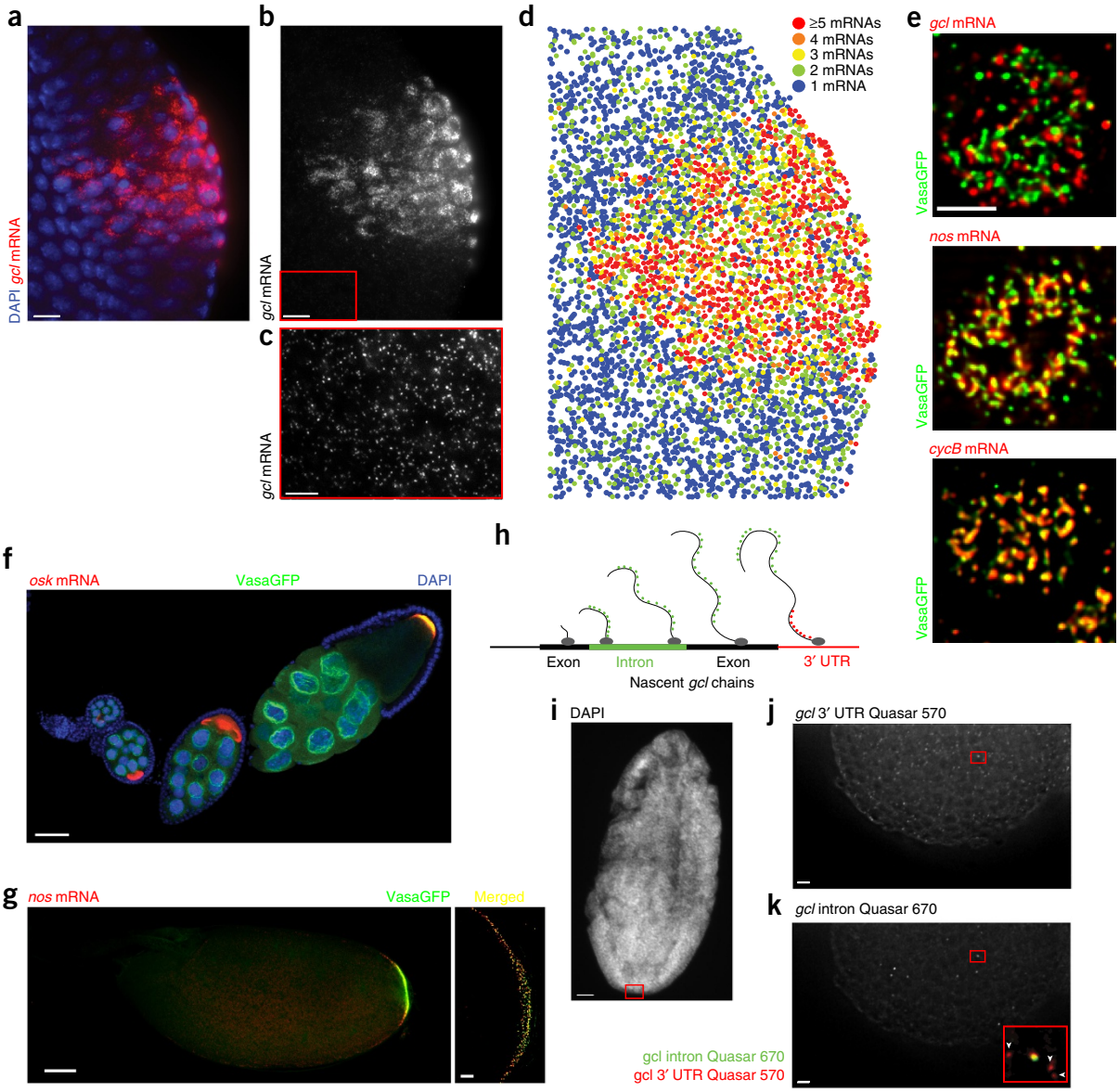

Figure 5 | smFISH enables spatial and quantitative characterization of gene expression in the fly tissue. (**a**–**c**) Images of *gcl* (red) localized at the posterior pole of an embryo forming crescents around the nuclei (labeled with DAPI, blue) of the newly formed primordial germ cells during the 13th nuclear cycle of the embryonic development (**a**). The inset marked with a red box in **b** highlights unlocalized, somatic *gcl* mRNA, which is further magnified and contrast-adjusted in **c**. (**d**) Heat map of *gcl* mRNA. In all panels, three consecutive *Z* planes (z = 400 nm) were maximally projected and subsequently analyzed. All images were acquired with a wide-field microscope in 3D and subsequently deconvolved using Huygens. (**e**) iSIM super-resolution imaging reveals a detailed spatial organization of germ-granule-localized *gcl*, *nos* and *cycB* mRNAs into homotypic mRNA clusters. Images show germ granules in the 10th nuclear cycle of early embryonic development. Images were acquired in 3D using iSIM and afterward deconvolved using Huygens. (**f**,**g**) Images demonstrating accumulation of *oskar* (*osk*) and *nanos* (*nos*) mRNA (red) at the posterior pole of a developing oocyte through oogenesis. Germ plasm was visualized with a Vasa transgene tagged with a GFP[5]. In **f**, the first ten oogenic stages are shown. In **g**, an oocyte in late oogenic stage 14 is shown. Nuclei in **f** were stained with DAPI (blue). Images in **f** and **g** (left panel in **g**) were acquired with a laser scanning confocal microscope. An image of the germ plasm shown under 'Merged' was acquired with iSIM. (**h**) A schematic depicting detection of a *gcl* mRNA transcription site using smFISH probes hybridizing to either *gcl* introns (green dots) or the *gcl* 3′ UTR (red dots). (**i**) An image of a DAPI-stained embryo to visualize nuclei. (**j**,**k**) Before being spliced, smFISH-hybridized introns (green) colocalize with the smFISH probes hybridizing to the *gcl* 3′ UTR (red) at the site of transcription (inset in **k**, yellow spot). Mature *gcl* mRNAs do not bind intronic probes (inset in **k**, arrowheads). Scale bars, 2.5 µm (**e**); 5 µm (**c**,**g** (right panel),**j**,**k**); 10 µm (**a**,**b**); and 50 µm (**f**,**g** (left panel),**i**).

Control experiments. A series of control experiments can be performed to assay the specificity of smFISH probe hybridization to the target mRNA. First, the hybridization specificity can be monitored by perturbing gene expression of a target mRNA and determining whether changes have occurred in the expression pattern of a target mRNA. For example, deletion of a gene should result in the specific loss in smFISH fluorescence of a target mRNA (**Fig. 2b**). Similarly, gene expression of a target mRNA can be reduced using fly lines expressing interfering mRNAs specifically designed to downregulate a target mRNA (available through the Bloomington Stock Center). In this case, one should expect a reduction in the number of fluorescently labeled target mRNAs in response to RNAi treatment.

A good way of determining whether the observed fluorescent signal originates from an mRNA hybridized with smFISH probes rather than from nonspecific background accumulation of smFISH probes is to colabel a target mRNA with two sets of spectrally distinct smFISH probe sets (**Fig. 2c**). Here, the two signals should colocalize and quantification of the number of mRNA molecules detected in both channels should give similar numbers. In addition, several sets of probes with increasing numbers of probes per set can be used to label a target mRNA. In this case, the average integrated fluorescence intensity of a single mRNA should increase but the absolute number of detected mRNAs should not change (**Fig. 4b**,**c**).

Up to 200 different mRNAs are estimated to be localized to the posterior pole in an early *Drosophila* embryo[16,68]; some of these have been shown to play important roles in embryonic pattern formation[69], and germ cell formation and specification[70]. We and others have characterized several maternally deposited mRNAs for their ability to localize to the posterior pole and found that unlocalized mRNA counterparts found in the somatic regions of the embryo are not packaged into larger ribonucleoprotein but are mostly found as single mRNAs[5,6]. For these mRNAs, the average integrated fluorescence intensity of a single mRNA can be unambiguously determined by fitting the distribution of integrated fluorescent intensities of all identified fluorescent spots to a Gaussian curve and calculating the peak of the Gaussian fit (**Fig. 4a**). An exception is *oskar* (*osk*) mRNA. Approximately 50% of unlocalized *osk* mRNA is present in clusters containing more than one *osk* mRNA[5,6,41,71], and the peak of the distribution of fluorescence intensity of spots containing a single *osk* mRNA is not easily identifiable. To troubleshoot quantification of clustered mRNAs such as *osk*, the mRNA expression levels determined for *osk* with smFISH can be compared with the relative mRNA expression levels determined by mRNA seq[5] (freely available through FlyBase[72]). For example, we have previously quantified mRNA levels of *ccr4*, *nos*, *gcl*, *pgc* and *osk* in early embryos laid by WT mothers and compared them with the relative mRNA levels determined for these mRNAs with mRNA-seq. The two measurements correlated exceptionally well ($r = 0.95$), indicating that mRNA levels, including *osk* mRNA levels, determined by smFISH were in a good agreement with relative mRNA expression levels determined for these mRNAs by RNA-seq[5]. This control provided a strong indication that appropriate spot-detection parameters were used during spot detection, which enabled identification of single smFISH-labeled *osk* mRNAs within a heterogeneous population of fluorescent spots containing multiple *osk* mRNA particles.

MATERIALS

REAGENTS

- Wild-type fruit flies (Bloomington *Drosophila* Stock Center) or the flies specified by the experiment
- Dry fine yeast (Lab Scientific, cat. no. FLY-8040-20F)
- Clorox Ultra Germicidal Liquid Bleach (Fisher Scientific, cat. no. 50371500)
- Heptane (Fisher Chemical, cat. no. O3008-4)
- 20% (vol/vol) Paraformaldehyde (Electron Microscopy Sciences, cat. no. 15713) **! CAUTION** Paraformaldehyde is a toxic cross-linking agent. Wear protective gloves and handle it in the fume hood. Discard according to the relevant environmental health and safety instructions.
- 100% (vol/vol) Methanol (Fisher Chemical, cat. no. A412-4)
- Triton X-100 (250 ml; Sigma-Aldrich, cat. no. T8787)
- Tween-20 (500 ml; Sigma-Aldrich, cat. no. P2287)
- 10× PBS, pH 7.4 (Thermo Fisher Scientific, cat. no. AM 9624)
- Proteinase K (Sigma-Aldrich, cat. no. 03 115879 001)
- Glycine (1 kg; Sigma-Aldrich, cat. no. G8790)
- 20× Saline-sodium citrate buffer (SSC) containing 3 M NaCl in 0.3 M sodium citrate (Thermo Fisher Scientific, cat. no. AM 9770)
- Deionized formamide (100 ml; Amresco, cat. no. 0606) **! CAUTION** Formamide is toxic and should be handled with protective gloves under a hood and discarded according to the relevant environmental health and safety instructions.
- Ribonucleoside vanadyl complex (VRC; New England BioLabs, cat. no. S1402S)
- smFISH probe mix (Biosearch Technologies, commercially available and custom designed Stellaris RNA FISH Probe Sets)
- Sheared salmon sperm DNA (Thermo Fisher Scientific, cat. no. AM9680)
- *E. coli* tRNA (Roche, cat. no. 1010951001)
- BSA (Roche, cat. no. 10711454001)
- Dextran sulfate sodium salt (Acros Organics, cat. no. 433241000)
- 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI; Roche, cat. no. 10 236 276 001)
- ProLong Gold Antifade Reagent (Invitrogen (Molecular Probes), cat. no. P36934)
- Prolong Diamond Antifade Reagent (Invitrogen (Molecular Probes), cat. no. P36961)
- Vectashield (Vector Labs, cat. no. H-1000)
- Immersion oil (see 'Equipment Setup' section)

EQUIPMENT

- 25 °C incubator (Thermo Fisher Scientific, Forma Environmental Chamber)
- Embryo collection cage (large: 8.75 cm × 14.8 cm; Flystuff.com, cat. no. 59-101) (**Fig. 1a**). The cage accommodates a Petri dish containing an apple juice plate that is securely attached to the cage with a plastic cap. The top of the cage is covered with a stainless-steel mesh to allow ample breathability while eliminating condensation **▲ CRITICAL** After each use, the cage must be cleaned with cold soapy water, not with ethanol or other alcohols.
- BD Falcon Petri dishes, 60 × 15 mm, for small apple juice plates (Fisher Scientific, cat. no. 25373-085)
- Wooden tongue depressors (Fisher Scientific, cat. no. 11-700-555)
- Plastic scintillation vials (Wheaton 20-ml HDPE Liquid Scintillation Vials with Polypropylene Caps; Fisher Scientific, cat. no. 986700)
- Nitex mesh (Dynamic Aqua-Supply, cat. no. NTX100)
- Dumont tweezers (no. 5, 11 cm, straight, 0.1 × 0.06-mm tips; Dumostar, cat. no. 500233)
- Dissecting microscope (Zeiss, model no. Stemi SV 11, equipped with a light source)
- Squirt bottle (VWR, cat. no. 16650-028)
- Pyrex glass flask (Corning, cat. no. 1395-500)
- Glass scintillation vial, Wheaton glass 20-ml scintillation vial with urea cap (Fisher Scientific, cat. no. 03-341-25J)
- Glass Pasteur pipette (Fisher Scientific, cat. no. 13-687-20C)
- 1.7-ml Super-clear microcentrifuge tubes (Crystalgen, cat. no. L-2052)
- NanoDrop spectrophotometer (Thermo Scientific, model no. ND-1000 UV/Vis)
- pH-indicator strips, pH 5.0–10.0 (colorpHast; EMD, cat. no. 9588)
- Vortex, standard mini vortex (VWR)
- Falcon 15-ml Conical Centrifuge Tubes (Fisher Scientific, cat. no. 14-959-53A)
- Tabletop centrifuge (Eppendorf, cat. no. 5424)
- 37 °C incubator (Isotemp standard lab incubator; Fisher Scientific)
- Microscope Slides (Fisher Scientific, cat. no. 12-544-2)
- Microscope coverslips, 22 × 22 mm, category 1.5 (thickness 0.16–0.19 mm; Fisher Scientific, cat. no. 12-544-D)
- Wide-field epifluorescence, confocal or instant structured illumination microscope (iSIM) with a super-resolution imaging system (see 'Equipment Setup' and Procedure Steps 27 and 28)

- Huygens deconvolution software (Scientific Volume Imaging, https://svi.nl/HuygensSoftware)
- Single-molecule detection algorithm (Airlocalize, can be downloaded from http://www.timotheelionnet.net/software/). See 'Equipment Setup' and Procedure Steps 29–42.
- SigmaPlot (Systat Software, https://systatsoftware.com/products/sigmaplot/)
- Dissection microscope (Zeiss, model no. SteREO Discovery.V8) equipped with a light source (Zeiss, model no. KL1500 LCD) and a flypad (Flystuff, standard Size (8.1 × 11.6-cm)) connected to a CO_2 tank to anesthetize the flies

REAGENT SETUP

▲ **CRITICAL** Maintain DNase- and RNase-free conditions and use DNase- and RNase-free reagents to prepare solutions used during smFISH hybridization.

Fruit flies Place between 200 and 400 2-d-old flies with a 2:1 female/male ratio in a bottle containing fly food (for a detailed protocol on how to prepare bottles containing fly food, refer to Ashburner[73]). Maintain the flies in a 25 °C incubator (or at the temperature specified by the experiment) for 2 d. Each morning and evening, provide additional dry yeast to the flies to stimulate egg laying. After 2 d, transfer the flies to the embryo collection cage mounted with an apple juice plate that contains a dollop of fresh yeast paste. Maintain the flies in the cage for 1 d at 25 °C (or the temperature specified by the experiment) so that they become used to the new food source and egg-laying environment.

Yeast paste Dissolve 30 g of fine dry yeast powder in 20 ml of H_2O. Stir frequently while incubating at room temperature (RT; 20–22 °C) for several hours and adjusting with H_2O until the yeast paste achieves a consistency similar to that of peanut butter. Store it at 4 °C for up to 2 weeks.

Apple juice plates These are Petri dishes filled half-way with agar mixed with apple juice. For a detailed protocol on how to prepare apple juice plates, refer to Ashburner[73]. A dollop of yeast paste is placed in the middle of the apple juice plate to provide food for the flies while they lay eggs on the surface of the apple juice plate. Apple juice plates are stored at 4 °C for 1 month. The yeast paste is placed on the juice plates immediately before use.

Fixative To prepare 10 ml of fixative, combine 2 ml of 20% (vol/vol) paraformaldehyde, 1 ml of 10× PBS and 7 ml of ddH_2O. ▲ **CRITICAL** Freshly prepare the fixative before use.

Saturated heptane Combine equal volumes of heptane and fixative (see above) in a glass flask to make a combined mixture of 10 ml. Close the flask and shake the mixture vigorously for 15 s. Let the solution settle into two phases. Repeat this mixing several times through the day and let it settle overnight (ON) to ensure that the heptane becomes saturated with paraformaldehyde. The saturated heptane is the upper phase in the 1:1 heptane/paraformaldehyde mixture. Wrap the flask in aluminum foil and store it at RT for up to 2 months.

Bleach solution Prepare 200 ml of 50% (vol/vol) bleach solution by mixing equal amounts of Clorox Ultra Germicidal Liquid Bleach and H_2O. Store the solution at RT for up to 2 weeks.

PBS–Tween(PBT) solution To make 1,000 ml of PBT, combine 100 ml of 10× PBS, 1 ml of Tween-20 and 899 ml of ddH_2O. Mix thoroughly and store the solution at RT for several months.

1× PBS solution To make 1,000 ml of 1× PBS solution, combine 100 ml of 10× PBS and 900 ml of ddH_2O. Store the solution at RT for up to several months. ▲ **CRITICAL** Maintain RNase-free conditions and use RNase-free reagents to prepare PBS solution.

3 µg/ml Proteinase K A 1 mg/ml stock proteinase K solution is prepared by dissolving 10 mg of lyophilized powder in 10 ml of ddH_2O; this solution can be stored at −20 °C for up to 12 months. To prepare 10 ml of working (3 µg/ml) proteinase K solution, combine 30 µl of stock (1 mg/ml) proteinase K solution with 10 ml of PBT. Freshly prepare the working (3 µg/ml) proteinase K solution.

2 mg/ml glycine solution Prepare 50 ml of 20 mg/ml glycine solution as a stock solution and keep it at 4 °C. Dilute tenfold with ddH_2O to obtain the working (2 mg/ml) glycine solution. Both solutions can be stored at RT for several months.

Prehybridization solution 10 ml of prehybridization solution contains 1 ml of 100% (vol/vol) deionized formamide (10% (vol/vol) final concentration), 1 ml of 20× SSC (2× SSC final concentration) and 8 ml of ddH_20. ▲ **CRITICAL** Freshly prepare the solution.

TE buffer To make 100 ml of TE buffer, pH 8.0, combine 1 ml of 1 M Tris-HCl (pH 8.0) and 0.2 ml of 0.5 M EDTA and make up the volume to 100 ml with ddH_2O. Adjust the pH to 8.0. Store the solution at RT for several months.

Competitor DNA To make 200 µl of 10 mg/ml of competitor DNA, combine 100 µl of 10 mg/ml sheared salmon sperm DNA with 100 µl of 10 mg/ml *E. coli* tRNA. The tRNA is prepared by dissolving 10 mg of powder in 1 ml of ddH_2O. Store it at −20 °C for up to a year.

smFISH probe mix Commercially available Stellaris FISH Probes were custom-designed against *gcl*, *nos* and *osk* by using the Stellaris RNA FISH Probe Designer (Biosearch Technologies) available online at http://www.biosearchtech.com/stellarisdesigner (**Supplementary Table 1**). Each smFISH probe mix is shipped as a dried set containing up to 48 individual smFISH probes hybridizing to different positions along the target mRNA (**Fig. 2a**). We most often use probes conjugated to CAL Fluor590, Quasar 570 or Quasar 670. Probes in each probe set are mixed in an equimolar ratio. The probe mix is resuspended in TE buffer to a final concentration of 40 ng/µl and can be stored at −20 °C for several years.

Hybridization mix To hybridize ~20 µl of embryos with a single smFISH probe mix, prepare 60 µl of hybridization mix. This mix contains 6 µl of 100% (vol/vol) deionized formamide (10% (vol/vol) final), 1 µl of competitor DNA, 2 µl of 40 ng/µl probe mix, 30 µl of 20% (vol/vol) dextran sulfate (10% (vol/vol) final concentration, see below), 5 µl of BSA (2 mg/ml final concentration), 5 µl of 20× SSC (2× SSC final concentration), 2.5 µl of 200 mM ribonucleoside vanadyl complex (10 mM final concentration) and 8.5 µl of ddH_2O. To hybridize multiple mRNA targets simultaneously using spectrally distinct probes, add 2 µl of 40 ng/µl probe mix for each target mRNA and adjust the total amount of ddH_2O accordingly. ▲ **CRITICAL** Freshly prepare the hybridization mix.

20% (vol/vol) Dextran sulfate To prepare 50 ml of 20% (vol/vol) dextran sulfate, combine 10 g of dextran sulfate with ddH_2O to obtain 50 ml. Store the solution at 4 °C for up to several months.

DAPI solution Prepare a 0.5 µg/ml DAPI solution in 1× PBS. Stir for several hours at RT in a flask wrapped in aluminum foil. Store the solution at 4 °C for up to several months.

EQUIPMENT SETUP

Embryo collection basket To make an embryo collection basket, cut the bottom off a plastic scintillation vial (**Fig. 1c**,**d**). Remove the plastic cap and cut a hole into the cap using a power tool or a flamed knife. Cut a circle the size of the plastic cap from the Nitex mesh and place it into the cap of the scintillation vial. Attach the cap with the mesh to the tube of the scintillation vial.

Wide-field epifluorescence microscope To acquire images with a wide-field epifluorescence microscope, an API DeltaVision personal DV system equipped with an Olympus IX-71 inverted microscope, Nomarski differential interference contrast optics and a Photometrics CoolSNAP HQ2 CCD camera with a pixel size of 6.45 µm (which meets the Nyquist criterion with a 60× magnification objective) was used. The microscope was guided by a SoftWorx suite. A xenon lamp and fiber optic module enabled uniform illumination of the field. A high-NA microscope objective such as the Olympus PlanApo N 60×/1.42 oil objective is desirable for collecting the maximum number of photons. This objective is optimized for imaging with no. 1.5 microscope coverslip with a working distance of 0.15 mm. To minimize spherical aberration, it is important to use an immersion oil that closely matches the refractive index of the objective lens. We used Olympus Type-F immersion oil (NA 1.516; Thorlabs, cat. no. MOIL-30). The customized DeltaVision fluorescence filter set, which includes a DAPI filter, a Tritc filter (for Quasar 570 and CAL Fluor590 detection) and a Cy5 filter (for Quasar 670 detection), maximizes emission collection and minimizes the spectral overlap in case of multicolor smFISH.

Scanning confocal microscope To acquire images using a laser scanning confocal microscope, a Zeiss LSM780 Axio Observer microscope equipped with a 32-channel GaAsp detector and two PMT detectors, a multiline 25 mW argon laser, a HeNe 633 laser, a 30-mW diode 405 laser, a DPSS 561-10 laser, and Plan-Apo 40×/1.4 Oil DIC (microscope coverslip no. 1.5 and working distance of 0.13 mm) and EC Plan-Neofluar 10×/0.30 (microscope coverslip no. 1.5 and working distance of 5.2 mm) objectives were used. We used Zeiss Immersol 518 F (NA 1.518) immersion oil (Fisher Scientific, cat. no. ISO 8036-1/2). The microscope was guided by a Zeiss ZEN system.

iSIM system To obtain super-resolution images of smFISH-labeled mRNAs, we used the iSIM[10,11], which was equipped with a 60× (note that the full magnification from sample to cameras was ~117×, however, because a 350-mm tube lens was used) NA 1.45 oil objective (Olympus) (with a working distance of 0.10 mm) and accompanying oil (type DF, refractive index 1.515, Cargille, cat. no. 410LDF-AB), and acquired images in green or red channels with 488-nm and 561-nm excitation lasers[5,10] and a filtering pump light with emission filters using a filter wheel (Sutter, FG-LB10-BIQ and FG-LB10-NW) and notch filters (Semrock, NF03-488E-25 and NF03-561E-25)[10,11]. These were placed immediately before the cameras to reject excitation laser light. Images were captured on a scientific CMOS camera with 6.5-µm pixels (pco, model no. edge 5.5)—the final imaging pixel size is 55.5 nm (=6.5 µm/117). Images are integrated from 5 to 20 20-ms 'sweeps' of the galvanometric mirror that scans the structured illumination through the sample, for an average exposure time of 100–400 ms per image. Stacks were assembled by acquiring data with a piezoelectric stage (Applied Scientific Instrumentation, 200-µm axial travel) affixed to the microscope body; typical step sizes range from 150 to 200 nm. We have previously fully described the alignment of the system[11]. Since its development in 2013, iSIM has become commercially available through VTi VisiTech International.

Deconvolution algorithm Microscopy images unavoidably suffer from artifacts such as blurring, which arises because of the optics of the microscope, and noise, which is typically dominated by Poisson statistics arising from the recording of fluorescence emissions. We use Huygens deconvolution software (v16.05; Scientific Volume Imaging) to reduce these imaging artifacts. Specifically, we use the iterative classic maximum likelihood estimation (CMLE) algorithm in Huygens, which is a restoration method that optimizes the likelihood of an estimate of an object given the measured image and PSF. The object estimate is in the form of a regular 3D image. The likelihood in this procedure is computed by a Quality Criterion under the assumption that the photon noise is governed by Poisson statistics. For this reason, it is well suited to increasing the signal-to-noise ratio and the effective resolution of an image, which facilitates the detection and counting of fluorescently labeled single mRNAs and, importantly, markedly increases the precision with which individual molecules can be localized (**Fig. 5a–d**). Settings used with the CMLE deconvolution algorithm are 50 iterations, a quality threshold of 0.01 and an SNR value of 40. We typically use the Huygens software to deconvolve our data, although a much simpler iterative Richardson–Lucy algorithm also worked well on our type of image data[5]. This algorithm is freely available and can be downloaded from https://code.google.com/archive/p/msim/source/default/source or http://bigwww.epfl.ch/deconvolution/deconvolutionlab2/.

Spot-detection algorithm Airlocalize When imaged with an epifluorescent, confocal or structured illumination microscope, mRNA molecules hybridized with smFISH probes appear as bright fluorescent spots against a uniform autofluorescent background. A spot-detection algorithm is used to automate spot detection and to measure the number, position and intensity of individual fluorescently labeled mRNAs in a 2D or 3D image. We use an algorithm called Airlocalize, which was developed in the MATLAB programming language (MathWorks)[9]. Download the Airlocalize script together with the MCRInstaller, which allows one to run a MATLAB algorithm without separately installing MATLAB onto the computer.

SigmaPlot SigmaPlot is a graphing and data analysis algorithm provided by Systat Software. It is used to calculate the average fluorescence intensity of fluorescently labeled mRNAs by fitting the distribution of the integrated fluorescence intensity of detected spots to a Gaussian curve, where the peak of the Gaussian curve determines the average fluorescence intensity of a single mRNA (**Fig. 4a**).

PROCEDURE

Collection and fixing of fly embryos for smFISH ● TIMING up to 1 d

1| Place 200–400 flies with a 2:1 female/male ratio into an egg-collection cage (**Fig. 1a**) mounted with an apple juice plate containing a dollop of yeast paste and prewarmed to 25 °C (or the temperature required for the specific experiment).

2| Place the egg-collection bottles into a 25 °C (or the temperature required for the specific experiment) incubator and allow the flies to lay eggs onto the apple juice plate.
? TROUBLESHOOTING

3| Remove the apple juice plate from the egg-collection cage (**Fig. 1b**), scrape off the yeast paste with a wooden tongue depressor and remove trapped flies with tweezers.

4| Fill the apple juice plate half-way with a bleach solution and incubate at RT for up to 2 min to dechorionate the embryos. Immediately proceed to Step 5.
▲ CRITICAL STEP The potency of bleach can vary. Monitor dechorionation on a dissecting microscope to prevent embryo damage by overexposure to bleach.
? TROUBLESHOOTING

5| Pour the embryos with the bleach solution into the egg-collection basket (**Fig. 1c**,**d**) and immediately wash the embryos with water using a squirt bottle. Wash the inner edges of the basket as well, so that all the embryos assemble in the center of the Nitex mesh.
? TROUBLESHOOTING

6| Remove the mesh with the dechorionated embryos from the egg-collection basket and use tweezers to place the mesh into a scintillation vial filled with 5 ml of saturated heptane and 5 ml of fixative.
! CAUTION Paraformaldehyde and heptane are toxic and should be handled with protective gloves under a fume hood and discarded according to the relevant environmental and safety instructions.

7| Shake the vial vigorously for 15 s. Let the vial stand at RT for 20 min. The embryos will settle at the interface between the lower paraformaldehyde and upper heptane layer.

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8| Using a glass pipette, remove the bottom paraformaldehyde layer. Remove as much fixative as possible without removing the embryos. Add 5 ml of 100% (vol/vol) methanol, cap the vial, shake vigorously for 15 s and let the embryos settle at RT to devitellinize them. Devitellinized embryos will settle at the bottom of the vial while embryos with a vitelline membrane will remain at the interphase between the two solutions.
! CAUTION Methanol is toxic when ingested or when in contact with skin or eyes. It is highly flammable. Handle it with protective gloves under a fume hood and discard according to the relevant environmental and safety instructions.
▲ CRITICAL STEP Only devitellinized embryos will hybridize smFISH probes.
? TROUBLESHOOTING

9| Collect and transfer the devitellinized embryos to a 1.7-ml tube using a glass Pasteur pipette or a cutoff pipette tip. Wash the embryos three times with 1 ml of 100% (vol/vol) methanol.
▲ CRITICAL STEP It is critical that a cutoff tip or a glass Pasteur pipette be used to preserve the embryos' integrity.
? TROUBLESHOOTING
■ PAUSE POINT Devitellinized embryos can be stored in 1 ml of 100% (vol/vol) methanol at 4 °C until further use (for up to several weeks).

Performing of smFISH ● TIMING 2–3 d
▲ CRITICAL *Drosophila* oocytes can also be stained following Steps 10–26 of this protocol. The procedure for dissection of female fruit flies to extract oocytes is shown in **Box 1**.
10| Transfer 20 µl of fixed embryos to a 1.7-ml tube.
▲ CRITICAL STEP Use a cutoff tip or a glass Pasteur pipette.
? TROUBLESHOOTING

11| Wash the embryos with 1 ml of 100% (vol/vol) methanol and incubate for 5 min at RT.

12| Wash the embryos twice with 1 ml of a 1:1 mixture of 100% (vol/vol) methanol/PBT, and incubate for 5 min at RT each time.

13| Wash the embryos twice with 1 ml of PBT and incubate at RT for 5 min each time.
■ PAUSE POINT Once rehydrated, the embryos can be stored in PBT for several hours at RT or for several days at 4 °C before continuing with Step 14.

14| Remove the PBT and postfix the embryos with 1 ml of fixative for 20 min at RT.

15| Remove the fixative and wash the embryos three times with 1 ml of PBT at RT, each time incubating for 2 min.

16| Remove the PBT, add 1 ml of proteinase K and incubate at RT for 13 min, inverting the tube frequently. Incubate the tube on ice for 1 h, inverting the tube every 15 min.
▲ CRITICAL STEP Proteinase K treatment is needed to ensure uniform smFISH probe penetration into the tissue and to ensure efficient probe binding to the mRNA. It is particularly recommended when an mRNA is assumed to be tightly packed with proteins (masked)[17] such that masking would preclude probe hybridization. The prolonged incubation on ice at low proteinase K concentrations ensures slow and uniform penetration and activity of proteinase K without overdigesting the tissue[13].
? TROUBLESHOOTING

17| Remove the proteinase K and inactivate the remaining proteinase K by washing the embryos with 1 ml of glycine and incubating at RT for 2 min. Repeat this step.
▲ CRITICAL STEP Thoroughly inactivate and remove the proteinase K to prevent overdigestion of the sample.
? TROUBLESHOOTING

18| Wash the embryos twice with 1 ml of PBT, incubating each time for 2 min at RT.

19| Remove the PBT and postfix the embryos with 1 ml of fixative for 20 min at RT.

20| Remove the fixative and wash the embryos five times with 1 ml of PBT at RT, incubating the embryos each time for 2 min to remove all traces of the fixative.

21| Completely remove the PBT, add 1 ml of prehybridization solution and incubate the embryos at RT for 10 min.
! CAUTION Formamide is toxic and should be handled with protective gloves under a fume hood and discarded according to the relevant environmental and safety instructions.
▲ CRITICAL STEP To ensure efficient probe hybridization and to minimize nonspecific probe binding, care must be taken to prepare a prehybridization solution with 10% (vol/vol) formamide and 2× SSC and to make sure that the hybridization of the sample with the probe mix will proceed at 37 °C.
? TROUBLESHOOTING

22| Remove the prehybridization solution and add 60 µl of hybridization solution containing the smFISH probe mix. Mix by gently flicking the tube with a finger, and incubate the tube, protected from light, at 37 °C ON in an incubator.
▲ CRITICAL STEP Do not spin the sample with a centrifuge. This will disintegrate the embryos. It is preferred to incubate the embryos in an incubator to prevent excessive evaporation of water into the cap of the tube. This will ensure that the concentration of formamide does not change during hybridization.
? TROUBLESHOOTING

23| Remove the hybridization solution and wash the embryos twice with 1 ml of prehybridization solution prewarmed to 37 °C, incubating each time for 15 min in a 37 °C incubator.
? TROUBLESHOOTING

24| Remove the prehybridization solution and wash the embryos twice with 1 ml of 1× PBS, incubating each time for 1 h at RT and protected from light.
? TROUBLESHOOTING

25| (Optional) To stain the nuclei, incubate with 1 ml of DAPI for 1 min at RT, protected from light. Afterward, remove the DAPI and incubate with 1 ml of 1× PBS for 5 min at RT.
? TROUBLESHOOTING

26| Transfer the embryos onto a microscope slide using a pipette with a cutoff tip. Then remove as much PBS as possible without drying the embryos. Add a few drops of mounting medium onto the embryos and cover them with a microscope coverslip. Seal the edges of the coverslip with nail polish or allow the mounting medium to cure according to the manufacturer's instructions.
▲ CRITICAL STEP Use a cutoff pipette tip or glass Pasteur pipette to transfer the embryos onto the microscope slide to prevent embryo disintegration. Do not dry the embryos, as this increases autofluorescence. Certain commercially available mounting media, such as Prolong Gold and Prolong Diamond, must be cured for ~24 h at RT in the dark before the samples can be imaged. Water-based mounting media such as Vectashield do not require this step, and samples can be imaged immediately after the microscope coverslip is sealed to the microscope slides with nail polish.
? TROUBLESHOOTING
■ PAUSE POINT Once mounting is completed, the slides can be stored at –20 °C for several months before being imaged.

Imaging of smFISH-stained embryos ● TIMING ~1–2 h
27| Embryos are imaged in 3D using a wide-field epifluorescence microscope, laser scanning confocal microscope or iSIM (Equipment Setup). Using a wide-field epifluorescence microscope or a laser scanning confocal microscope, 3D images of embryos are acquired with a 200- to 400-nm *Z*-step size, spanning a 2.5- to 5.0-µm depth. When iSIM is used, 3D images of embryos are acquired with a finer *Z* step (because of the superior optical sectioning of this approach), typically with a 150- to 200-nm *Z* step size, spanning a 2.5- to 5.0-µm depth. Whenever possible, exposure times, laser powers, and numbers of scans and sweeps are chosen such that the range of fluorescence intensities of collected light occupies 90% of the dynamic range of the camera or the detector while also making sure to minimize bleaching of the sample.
▲ CRITICAL STEP Long exposure, high laser powers, and multiple scans and sweeps can rapidly bleach the fluorescent signal. To increase the fluorescence intensity of a single mRNA during imaging, it is better to increase the number of probes hybridizing to an mRNA or to choose probes coupled to a brighter and more photostable dye, rather than increasing the exposure time or laser strength during imaging.
? TROUBLESHOOTING
■ PAUSE POINT Proceed to Step 28 or pause until time permits continuing with image preparation and analysis.

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Deconvolution of 3D image stacks ● TIMING ~1–2 h

28| Deconvolve the 3D-acquired wide-field epifluorescence or iSIM images using the Huygens deconvolution software, Richardson–Lucy algorithm or similar software. If the images were acquired with a laser scanning confocal microscope, proceed to Step 29.
? TROUBLESHOOTING
■ **PAUSE POINT** Proceed to Step 29 or pause until time permits continuing with image preparation and analysis.

smFISH detection and counting using Airlocalize ● TIMING ~1 d, scales with proficiency of the user

29| Launch Airlocalize.
? TROUBLESHOOTING

30| Select 'File Mode'. Choose '2D single image file'.
▲ **CRITICAL STEP** As a proof of principle, we provide here a step-by-step protocol of detection and counting of smFISH-labeled mRNAs in a single 2D image. However, a single 3D image file can be similarly analyzed. To further automate and speed up quantification, multiple 2D or 3D image files can be analyzed in a batch mode option by choosing the '2D or 3D images, batch mode' option.
? TROUBLESHOOTING

31| Select 'Detection and Quantification' parameters (**Fig. 3a**). Define the spatial characteristics of the PSF, whether a ROI or an entire image is analyzed, and define how the detection threshold is determined. *A priori* knowledge of the PSF is not required: the PSF parameters can be estimated from the single-mRNA data within the Airlocalize interface by checking the 'Set Manually' radio button within the 'PSF width (Pix)' box (Steps 34–37).
? TROUBLESHOOTING

32| Select the 'Output Image of Spots' option if an image of detected smFISH-labeled mRNAs is desired (**Fig. 3g**,**h**).
? TROUBLESHOOTING

33| The 'Advanced Parameters' section consists of software parameters that typically do not need to be modified; the defaults are well adapted to most single-molecule detection applications. These parameters include the maximum number of spots to consider in the image, the minimum distance allowed between spots (spots closer than this distance will be reduced to a single spot), the shape of the band-pass filter ('High pass Length Cutoff', 'Low pass Length Cutoff', 'Cutoff Width'), the PSF model used ('PSF type', default: 'Gaussian Kernel Integrated over the pixel/voxel'), the fitting algorithm ('Quantification Method', default: 'Gaussian Mask'), the size of the region around each spot used to compute position and intensity ('Local Fit Size'), the thickness of the area surrounding the fitting region used to estimate the background ('Local Background Thickness'), the algorithm used to compute the background value (default: 'local plane') and the maximum number of iterations allowed for the mask to converge. Once all the parameters are defined, select 'next'.
? TROUBLESHOOTING

34| Define the shape of a 3D Gaussian mask by executing Steps 34–37. This mask will be used to quantify the integrated fluorescence intensity of smFISH-labeled mRNAs (**Fig. 3b**). Scroll over the image located in the left panel and choose a representative fluorescent spot of the smFISH-labeled mRNA. Find the center of the spot and left-click to select the spot (a close-up of the image is located in the bottom right panel in the software, which helps in selecting the center of the fluorescent spot) (**Fig. 3c**). A 2D fluorescence profile of the spot will appear in the upper right two panels (**Fig. 3d**), demonstrating the distribution of the fluorescence intensity of the spot (*y* axis; arbitrary units (a.u.)) along the *x* (*x* axis in pixels; upper panel) and along the *y* (*x* axis in pixels; lower panel) axes in an image.
? TROUBLESHOOTING

35| Click 'local gaussian fit' (**Fig. 3c**) to calculate the shape of the 3D Gaussian mask overlaying the fluorescent spot (**Fig. 3d**). The shape of the calculated mask will be indicated as a red curve overlaying the recorded fluorescence intensity profile of the fluorescent spot (marked with a blue line) (**Fig. 3d**).
? TROUBLESHOOTING

36| Click 'Record Fit Results'.
▲ **CRITICAL STEP** A good PSF is critical in making sure that the Gaussian mask will optimally fit the fluorescently labeled mRNAs, calculate their integrated fluorescence intensity and determine the center of mass of the smFISH-labeled mRNA with a subpixel resolution. Therefore, it is important that the fit (red curve) deviate minimally from the measured data (blue curve) and thus appropriately describe the PSF of the fluorescent spot.
? TROUBLESHOOTING

37| Repeat Steps 34–36 on five to ten individual spots to obtain an average 3D Gaussian mask, which will be used to detect and quantify the fluorescence intensity of smFISH-labeled mRNAs in subsequent steps. Click 'done'.
? TROUBLESHOOTING

38| Specify the fluorescence intensity threshold for detection of smFISH-labeled mRNAs (**Fig. 3e**). The pixels whose fluorescence intensity is above the specified threshold are marked in red, pixels of the input image are marked in green and colocalized pixels are yellow.
? TROUBLESHOOTING

39| Chose 'Done'. Airlocalize provides three files important for further analysis and stores them in the folder from which the analyzed image originates. The first is a TIF file corresponding to the 'Output Image of Spots'. This is an image in which the *x*, *y* and *z* positions of the centers of mass (the peaks of fluorescence intensity) of all detected fluorescent spots in an original image (**Fig. 3g**) are marked in red (**Fig. 3h**). The second is a localization (a .loc file or a .loc3 file, for 3D images, consisting of ASCII text) file that lists the spatial and quantitative information for all detected fluorescent spots. On this list, each spot is listed in a row, with its position specified with a subpixel resolution (*x*, *y*, *z* in units of pixels in columns A, B and C, respectively) and its integrated fluorescence intensity (a.u.) encompassed by the 3D Gaussian mask provided (column D). The third (a .par file, consisting of ASCII text) file lists all the parameters specified during the analysis.
? TROUBLESHOOTING

40| Using SigmaPlot or a similar graphing and data analysis algorithm, plot the distribution of integrated fluorescence intensities of all detected mRNAs as a histogram. To calculate the average fluorescence intensity of a single mRNA, fit the data (black dots) to a Gaussian function (red curve) (**Fig. 4a**) using a curve-fitting algorithm such as SigmaPlot. The peak of the Gaussian fit determines the average florescence intensity of a single smFISH-labeled mRNA.
▲ CRITICAL STEP When a large fraction of mRNAs is in clusters, a Gaussian fit with a single peak to determine the average fluorescence intensity of a single mRNA is not always possible. In this case, two (or more) populations of fluorescent spots that contain two (or more) mRNAs per spot will be revealed in the graph. Here, a Gaussian fit with two (or more) peaks should be used; otherwise, an average spot intensity will be skewed if these distinct mRNA populations are combined into one average.
? TROUBLESHOOTING

41| To determine the absolute number of mRNAs within a cluster, the cumulative fluorescence intensity of each fluorescent cluster is normalized against the average fluorescence intensity of a single mRNA determined by the Gaussian fit (**Fig. 5a–d**). Clusters with a normalized fluorescence intensity ranging between 0.51 and 1.50 are considered to contain 1 smFISH-labeled mRNA; those with a normalized fluorescence intensity ranging between 1.51 and 2.50 are considered to contain two smFISH-labeled mRNAs; those with a normalized fluorescence intensity ranging between 2.51 and 3.50 are considered to contain three smFISH-labeled mRNAs; and so on (**Fig. 5d**). To demonstrate the spatial distribution of mRNAs within the embryo as a heat map, the average integrated fluorescence intensity of a single mRNA—in this case a single *gcl* mRNA—was first determined (**Figs. 3** and **4a**), after which this value was used to calibrate the intensities of clustered *gcl* spots at the posterior pole. During spot detection, Airlocalize determines the position of each single mRNA and mRNA cluster in 2D and 3D with subpixel resolution. These coordinates are then used to plot the spatial distribution of individual mRNAs and clusters in the embryo and to create the heat map.
? TROUBLESHOOTING

42| When analyzing multiple images acquired in the same condition, it is critical to ensure consistency of the detection parameters. The number of spots detected, as well as their intensity, can be sensitive to the choice of PSF size and threshold. To ensure consistent analysis, the PSF size and threshold can be set manually on an initial pilot image; the values can then be extracted from the resulting .par file (listed under 'sigma_xy' or 'sigma_z' (for 3D images) and 'thresh.level') and typed into the corresponding dialog boxes (**Fig. 3a**) when analyzing subsequent images or launching a batch analysis. The 'Set Manually' buttons must be unchecked in this case in order to bypass Steps 34–38 and ensure consistent settings across images.
? TROUBLESHOOTING

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

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TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	Flies lay few eggs	(i) Not enough flies in the cage	(i) This can be fly-line-dependent, as some flies are not as fecund. Add more flies to the cage
		(ii) Apple juice plates are cold	(ii) Apple juice plates must be prewarmed to 25 °C (or the temperature at which the eggs are collected), as the cold shock reduces egg-laying
		(iii) No surface for flies to lay eggs	(iii) Place the yeast paste in the middle of the plate to allow the flies to lay eggs away from food or reduce the number of flies. Add filter paper to the cage, so that flies do not get stuck on the plate
		(iv) Flies are too old	(iv) Use flies that are at the peak of their fecundity (3–7-d-old flies)
		(v) Flies are not acclimated to the apple juice plate	(v) Acclimate flies to apple juice plates by keeping them in an egg-collection cage for one day before collecting eggs
		(vi) Flies are not well fed	(vi) Feed the flies well with yeast paste to stimulate egg production
		(vii) Eggs are not collected at the appropriate time of the day	(vii) Flies grow on a 12-h light cycle. Flies lay eggs best toward dusk and worst at dawn. Collect later in the day or use an incubator with a controlled 12-h light cycle
4, 5	Embryos disintegrate or have impaired morphology	Bleach times are too long or the potency of the bleach is too high	As a rule of thumb, the embryos are dechorionated after they detach from an apple juice plate and start floating in the bleach solution. Monitor the efficiency of dechorionation under the dissecting microscope. When the dorsal appendages have dissolved in 80% of the embryos (~2 min), remove the bleach and wash the embryos with water
8, 9	Younger embryos did not devitellinize	Methanol cracking was used to devitellinize embryos	Younger embryos found in nuclear cell cycles 1–10 do not devitellinize efficiently using methanol cracking. Try hand-devitellinization instead
	Embryos of various stages poorly devitellinize	Fixative was not completely removed	It is important to remove as much fixative as possible, as it inhibits devitellinization of the embryos. When fixing larger numbers of embryos, larger vials with larger methanol/heptanol volumes are preferred. By increasing the methanol surface, the embryos will crack their vitelline membrane more readily. Remove the devitellinized embryos from the bottom of the vial and add 5 ml of fresh 100% (vol/vol) methanol and repeat methanol cracking
9,10, 22, 26, 27	Embryos disintegrate or have impaired morphology	(i) Too much mechanical force was applied when transferring the embryos	(ii) Use only cutoff pipette tips or glass Pasteur pipettes to prevent shearing the embryos. Do not spin embryos with a centrifuge. This will disintegrate the embryos, particularly after they have been treated with proteinase K and formamide
		(ii) Embryos are insufficiently fixed	(ii) Increase the duration of fixation with fixative to 45 min. Autofluorescent background does not increase significantly with longer fixation times. When fixing for 20 min with 4% (vol/vol) paraformaldehyde, heptane must first be saturated with 4% (vol/vol) paraformaldehyde to ensure even and fast fixation. Saturated heptane must be prepared at least one day before and must be frequently shaken throughout the day to promote saturation
9, 10, 26	Embryos stick to Pasteur pipettes and pipette tips	Embryos are resuspended in 1× PBS	Wet the glass Pasteur pipette or cutoff pipette tip with methanol or PBT before pipetting the embryos. Alcohols and detergents prevent embryos from sticking to glass and plastic. If embryos are sticking too much, PBS can be replaced with PBT

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
9, 21–42	Autofluorescence is too high	(i) Embryos have been stored for too long	(i) Avoid using embryos that have been stored for too long, as prolonged storage can increase autofluorescence
		(ii) Embryos became dried during smFISH labeling, leading to increased autofluorescence	(ii) Keep the embryos hydrated during the smFISH labeling
		(iii) Staining with DAPI was excessive	(iii) Reduce the time and the concentration of DNA staining with DAPI. DAPI has a broad emission spectrum that can cause bleed-through of signal into other channels when images of multiple channels are captured simultaneously
		(iv) The embryos' autofluorescence is too high	(iv) Use TrueBlack Lipofuscin Autofluorescence Quencher (Biotium) to reduce the autofluorescent background
		(v) Embryos were stored in 1× PBS	(v) Store embryos in 100% (vol/vol) methanol rather than 1× PBS or PBT. Methanol perforates the embryos and washes some autofluorescence away
16, 17	Embryos disintegrate or have impaired morphology	Embryos are overdigested	The duration of proteinase K treatment is too long or the concentration of proteinase K is too high. Adjust the time and concentration of proteinase K treatment
	Oocytes disintegrate or have impaired morphology	Oocytes are overdigested	Oocytes can be susceptible to proteinase K overdigestion and can lose their morphology during treatment. The duration and the potency of the proteinase K treatment can be reduced or omitted during smFISH staining of oocytes
21–24 27, 29–42	No or low smFISH signal	The number of smFISH probes is insufficient to detect single mRNAs	Design more smFISH probes against the target mRNA. The probes can cover 5 and 3 untranslated regions (UTRs), as well as the ORF
		Inappropriate formamide concentration	Formamide concentrations >10% (vol/vol) prevent probe binding. Adjust the formamide concentration to a final concentration of 10% (vol/vol). It is preferable to incubate the embryos in an incubator to prevent excessive evaporation of water into the cap of the tube. This will ensure that the concentration of formamide does not change during hybridization (an increase in the formamide concentration could prevent efficient probe hybridization)
		Inappropriate hybridization temperature	Temperature during hybridization is >37 °C, which prevents probe binding
		Inappropriate formamide pH	The pH of the formamide is too acidic or the formamide is not deionized. When exposed to air, formamide becomes acidified and quenches (inhibits) fluorescence of certain organic dyes such as Quasar 670. Prepare aliquots of fresh formamide in 1.7-ml tubes and store them at –80 °C until use. Over time, monitor the quality of the stored deionized formamide using a pH strip. A pH between 7.5 and 8 indicates high-quality deionized formamide
		smFISH probes did not penetrate the embryo	It is necessary to incubate embryos in 100% (vol/vol) methanol before hybridization for at least a couple of hours to ensure that smFISH probes will penetrate the embryo during hybridization. If methanol is avoided, 1× PBS containing 1% (vol/vol) Triton X-100 must be used instead to perforate the embryos. PBT containing 0.1% (vol/vol) Tween-20 does not have sufficient detergent strength to perforate the embryos. Alternatively, embryos are not devitellinized. Repeat methanol cracking

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
		Dyes coupled to smFISH probes are not bright or photostable	Choose smFISH probes coupled to a bright and photostable dye. We most often use probes labeled with CAL Fluor590, Quasar 570 and Quasar 670. Quasar 670 is bright; however, when imaged with a laser scanning confocal microscope, Quasar 670 does not excite well and bleaches rapidly. It is also sensitive to the pH of the formamide (see above). Quasar 570 is not as bright as Quasar 670 or CAL Fluor 590
		Proteinase K treatment did not work	Prepare fresh proteinase K solution
		DNases and RNases contaminated the smFISH probe mix and/or sample and degraded probes and/or mRNAs in the sample	Although we have not experienced signal loss because of smFISH probe or mRNA degradation during our smFISH staining (smFISH probes are DNA primers that are fairly resistant to degradation, whereas mRNAs in the embryos are fixed before hybridization and samples are treated with VRC RNase inhibitor during hybridization; both treatments stabilize mRNAs during hybridization), care must be taken by using DNase- and RNase-free reagents and laboratory practices
26, 27	Oocytes disintegrate or have impaired morphology	Too much mechanical force was applied when transferring the oocytes	Use only cutoff pipette tips or glass Pasteur pipettes to prevent shearing of the oocytes. Do not spin oocytes with a centrifuge, as they will disintegrate
22, 23, 24, 27, 29–42	Background smFISH fluorescence is too high	Too much smFISH probe was used	Reduce the amount of probe used
		Hybridization times were too long	Implement shorter hybridization periods
		The frequency and the duration of washes was too short	Increase the frequency and the duration of washes with the prehybridization solution and PBS
		The concentration of BSA or DNA competitor was too low	Increase the concentration of BSA and competitor DNA
		Inappropriate formamide concentration	Increase the formamide concentration to >10% (vol/vol) to prevent nonspecific probe binding
		Inappropriate hybridization temperature	Decrease the hybridization temperature during hybridization to 37 °C to prevent nonspecific probe binding
24, 26, 27, 29–42	Fluorescent signal bleaches	An inappropriate mounting medium was used	Use an appropriate mounting medium that is an efficient scavenger of oxygen radicals. These can cause bleaching of the fluorophores. We regularly use Vectashield, Prolong Gold and Prolong Diamond
		Exposure time was too long and/or the power of the light or laser was too high; the number of sweeps was too high	Reduce the exposure time and/or the power of the light (wide-field microscope), reduce laser power (confocal microscope, iSIM), reduce the number of scanned lines (confocal microscope), reduce the number of sweeps (iSIM) and reduce the number of optical *Z* sections (all microscopes)
		Dyes coupled to smFISH probes are not bright or photostable	Use a photostable fluorophore. CAL Fluor 590 is very photostable, whereas Quasar 690 bleaches more rapidly, particularly when a laser scanning confocal microscope is used in which a focused, high-intensity laser line excites the fluorophores. To reduce the rate of photobleaching during imaging, add 1 mM Trolox antioxidant to the PBS washes (Step 24). Curing mounting media might not diffuse deeply into the embryo before they solidify to prevent photobleaching. By adding Trolox during PBS washes, the embryo is thoroughly perfused with the anti-bleaching reagent

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
27	No or low smFISH signal	Imaging of mRNAs too deep in tissue	The smFISH signal might be visible at the surface of the embryo but not deeper in tissue. This occurs because of photon loss when emitted light scatters during image capture
	Samples move during imaging	A water-based mounting medium was used	Use a curing mounting medium. We use Prolong Gold and Prolong Diamond. These media cross-link and fix the embryos on the coverslip and the embryos do not move during the imaging
27, 29–42	Reduced resolution of iSIM	Imaging too deep	The optimal imaging depth of an iSIM microscope is sample-dependent, but typically no more than 50–60 µm in transparent tissue (and in *Drosophila* tissue when using a 60× 1.45 NA objective and imaging mRNA-bound germ granules located immediately beneath the surface of the embryo's posterior pole, it is no more than 40 µm from the coverslip). Ensure that the working distance of the objective is sufficient to image at the chosen depth from the coverslip
		Wrong microscope coverslip	It is important that the coverslip is a no. 1.5 (thickness 0.16–0.19 mm)
		The refractive index of the mounting medium does not match the refractive index of glass, or the objective	We typically use Vectashield, Prolong Gold and Prolong Diamond to mount our samples; it is therefore important to select an oil objective designed to work with an oil of similar refractive index
28	Deconvolution artifacts	Acquired images are not suitable for deconvolution	3D images acquired on a wide-field microscope or iSIM are typically deconvolved before being analyzed to count single mRNAs. There are no problems intrinsic to wide-field or iSIM deconvolution. 3D stacks acquired on a laser scanning confocal microscope are typically not deconvolved. If the fluorescent signals of smFISH-labeled mRNAs are strong enough, images can be analyzed without prior deconvolution. In this case, deconvolution facilitates only the detection and counting, as it makes fluorescently labeled mRNAs brighter. However, because of an increase in contrast and signal assigned to the spot, properly applied deconvolution markedly increases the precision with which individual fluorescent molecules can be localized. Thus, when one wishes to determine where in a 3D space particular mRNAs lie (with a subpixel resolution), deconvolution of image stacks before analysis is strongly advised
		Deconvolved images contain ringing artifacts (black regions in images or in axial reslices of volume)	Check that the parameters of the experiment properly match the settings in the deconvolution software. Magnification and axial step size are particularly important. Also check that the axial step size is set to properly sample the axial extent of the PSF (ideally at Nyquist sampling size or better)
		Deconvolved images display degraded resolution relative to raw data	Verify that the model of PSF used in the deconvolution software (e.g., Gaussian, theoretical) well approximates the experimental PSF. If not, the model of the PSF needs to be revised for better agreement, or an experimental PSF should be used in the deconvolution software. If the latter option is pursued, ensure that the PSF is as noise-free as possible
31	Spot detection with Airlocalize is slow	The duration of spot detection analysis increases with increasing data set size and can become very long for large image volumes	To circumvent imaging and analysis of very large image data sets, a 3D ROI of a known volume can be used as a proxy for the concentration of unlocalized mRNAs across the entire embryo. Single mRNAs are then counted in the 3D image stack as described in the Steps 29 through 41 and the concentration of mRNAs determined by extrapolating to the known embryo volume[5]. Alternatively, data sets can be truncated using standard image analysis tools (e.g., Fiji) before Airlocalize processing

(continued)

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TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
31	Inefficient spot detection	Inappropriate definition of PSF for spot detection	Visual aids (profile along *x*, *y*, *z* in **Fig. 3d**,**f**) can be checked to ensure that the software appropriately fits the intensity of the spots in the image. The program can fail to converge if the spots are extremely dim or the background is very high. In this case, the PSF parameters can be computed using diffraction-limited beads imaged under identical conditions (objective, filter sets)
39, 41, 42	Detection algorithm over/undercounts	(i) Threshold parameters are not optimized	(i) Fluorescence intensities of the fluorescent mRNAs/probes change with the exposure time. For each imaged condition, determine the threshold parameters separately. Threshold parameters will determine which fluorescent spots will be included as single mRNAs; optimizing threshold parameters is critical to achieving successful single mRNA analysis. If the threshold is set too high, the algorithm will detect only the brightest fluorescent spots (those that 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 fit toward lower fluorescence intensities. It is critical to use biological controls to confirm selectivity (using fluorescent probes that have no reactivity with any mRNA in the tissue—e.g., using a knockout sample; **Fig. 2d**) and sensitivity (by comparing absolute counts to known abundance calculated by other means (Anticipated Results), or performing a double-labeling experiment, **Fig. 2e**). The threshold can be set as absolute or as a standard deviation. An absolute threshold (i.e., threshold in units of fluorescence counts) should be used when analyzing FISH data. It is critical to analyze all images from the same data set with the same threshold value to avoid biases
		(ii) Spots are difficult to observe when running Airlocalize	(ii) Adjust minimal (min) and maximal (max) fluorescence intensity (Int.) settings located above the image panel

● TIMING

Steps 1–9, collection and fixing of fly embryos for smFISH: up to 1 d
Steps 10–26, performing of smFISH: 2–3 d
Step 27, imaging of smFISH-stained embryos: ~1–2 h
Step 28, deconvolution of 3D image stacks: ~1–2 h
Steps 29–42, smFISH detection and counting using Airlocalize: ~1 d, scales with the proficiency of the user
Box 1, preparation of late-stage *Drosophila* oocytes for staining with smFISH probes: up to 1 h

ANTICIPATED RESULTS

This protocol can be used to stain and image *Drosophila* embryos, followed by the analysis of 3D images with Airlocalize to detect and count single smFISH-labeled mRNAs within a volume. Labeled mRNA molecules are observed as discrete bright fluorescent spots surrounded by a uniform autofluorescent background of lower intensity (**Figs. 2a**,**c** (middle), **3g** and **5c**,**d**). The average integrated fluorescence intensity of a single smFISH-labeled mRNA scales linearly with the number of smFISH probes used in the hybridization mix (**Fig. 4b**,**c**); however, the number of detected mRNAs per embryos does not change (**Fig. 4b**,**c**). This experiment provides a good control to determine whether the observed fluorescent signal originates from an mRNA hybridized with smFISH probes or from nonspecific accumulation of smFISH probes. It is also important to note here that the accuracy in detecting the true number of mRNAs increases with the number of smFISH probes used (note the diminishing error bars in **Fig. 4c**). To bypass issues associated with analyzing large image data sets, a representative 3D ROI of a known volume can be analyzed to serve as a proxy for the concentration of unlocalized mRNAs across the entire embryo. After determining the volume of a representative 3D ROI within an embryo[74] and the number of mRNA molecules within this ROI (and hence the mRNA concentration), information can be extrapolated to the entire embryo[5].

Spatial distribution of mRNAs within germ granules in early *Drosophila* embryos

Once an average integrated fluorescence intensity of a single smFISH-labeled mRNA is determined (**Fig. 4a**), it is used to calibrate the fluorescence intensities of all fluorescent spots identified by the spot-detection algorithm in an image and to determine the absolute number of mRNAs clustered in each fluorescent spot. mRNA clusters whose normalized fluorescence intensity ranges between 0.51 and 1.50 are considered to contain 1 smFISH-labeled mRNA; those whose normalized fluorescence intensity ranges between 1.51 and 2.50 are considered to contain two smFISH-labeled mRNAs; those whose normalized fluorescence intensity ranges between 2.51 and 3.50 are considered to contain three smFISH-labeled mRNAs; and so on (**Fig. 5a–d**)[5]. A particular advantage of smFISH is that the position of a labeled mRNA in 2D and 3D can be determined with a subpixel resolution simply by measuring the center of mass of an mRNA hybridized with smFISH probes (**Fig. 3b**,**c**). The accuracy with which the center of mass can be measured is determined by the signal-to-noise ratio of an image. We have empirically determined that for mRNA clusters localized to *Drosophila* germ granules in an early embryo and imaged with iSIM[5] the precision with which we are able to determine the position of smFISH-labeled mRNA clusters ranges between 9.1±2.9 nm and 14.7±1.6 nm. The subpixel position of each mRNA can be combined with the number of mRNAs located in each fluorescent spot to spatially quantify gene expression in the fly tissue in 3D. An example of such an analysis is provided in **Figure 5a–d**, which shows the distribution of *gcl* mRNA at the posterior pole during the 13th nuclear cycle of embryonic development in WT embryos. Approximately 3% of maternally deposited *gcl* mRNA is localized to the posterior pole of an embryo, where multiple *gcl* mRNAs group to form homotypic *gcl* mRNA clusters that have higher fluorescence intensities and therefore contain multiple *gcl* mRNAs in a diffraction-limited volume (**Figs. 2a** (bottom) and **5a–d**)[5]. The *gcl* mRNA heat map depicted in **Figure 5d** demonstrates that outside of the posterior pole the majority of *gcl* transcripts are found as single mRNAs and only organize into clusters once they are localized at the posterior pole, consistent with the literature[5,6]. At the posterior pole, localized mRNAs interact with proteins assembled into membrane-less organelles called germ granules[5,6,68,75–77]. When smFISH was combined with iSIM, we revealed that *gcl*, *nos* and *cycB* form homotypic mRNA clusters that are asymmetrically distributed within germ granules. Specifically, *gcl* clusters are located at the periphery of the granule, *cycB* clusters are located in the center of the granule and *nos* clusters occupy an intermediate space between *gcl* and *cycB* clusters[5] (**Fig. 5e**). Such spatial sensitivity cannot be obtained with conventional wide-field epifluorescence or laser scanning confocal microscopy.

mRNA localization during *Drosophila* oogenesis

In addition to studying early embryogenesis, our staining, imaging and spot-detection protocol can also be implemented to image mRNA localization during other *Drosophila* developmental stages such as oogenesis (**Fig. 5f**,**g**). Historically, detection of mRNAs during late fly oogenesis (stages 11–14) using whole-mount *in situ* studies has been challenging because of poor probe penetration into tissue during hybridization. The vitelline membrane, which forms by the end of oogenic stage 10 (ref. 78), represents an impenetrable barrier for the longer FISH probes used in whole-mount *in situ* studies and prevents access of these probes to target mRNAs. To ensure that long RNA probes hybridize to transcripts expressed in oocytes during late oogenesis, the vitelline membrane must be removed before staining[15]. This is typically achieved by rolling of fixed oocytes on a 'rough' surface such as the frosted portion of the microscope slide[15]. This process, however, is laborious and can damage oocytes when excessive mechanical force is applied during rolling. Short smFISH probes, on the other hand, easily penetrate oocyte tissue even without rolling and readily hybridize to target mRNAs during all oogenic stages[6,8,20,21] (**Box 1**, **Fig. 5f**,**g**).

Identifying active sites of transcription in *Drosophila* embryos

The protocol described here can also be implemented to detect active sites of transcription in *Drosophila* embryos undergoing zygotic gene expression (**Fig. 5h–k**). It has been shown that active transcription sites are often bigger and brighter than single cytoplasmic transcripts or mature nuclear transcripts that have diffused away from the transcription site, indicating that multiple RNA polymerases II were actively transcribing a gene at the time of fixation and smFISH probe hybridization[1,22,23,79,80]. When several nascent mRNA chains are associated with an active site of transcription, they accommodate binding of multiple smFISH probes, resulting in a high smFISH probe intensity at an active transcription site (**Fig. 5h**). For this reason, one can identify active sites of transcription simply by looking for the brightest nuclear signal, as has been shown in *Drosophila* embryos, mammalian cells and tissue, and budding yeast[4,7,22,23,44,51,80–83].

As the transcription proceeds, the intronic regions in the pre-mRNA are also transcribed and can be detected by designing smFISH probes that hybridize specifically to the intronic regions in the unspliced nascent mRNA. Because a typical *Drosophila* gene contains multiple introns and because an entire intron must be transcribed before it can be spliced, some proportion of the nascent mRNAs will contain introns and bind intronic smFISH probes. On the other hand, smFISH probes targeting mRNA exons will label both nascent and spliced transcripts. The latter are generally smaller and have lower total fluorescence than active transcription sites (**Fig. 5h**,**k**, red dots)[4,7,22,23,44,51,80,81,84]. Larger size, increased total fluorescence and the ability to bind smFISH probes designed against the intronic pre-mRNA regions are suitable markers that distinguish active transcription sites from mature freely diffusing nuclear or cytoplasmic transcripts (**Fig. 5h**,**k**, green dots)[1,32,33,51,84,85].

However, it must be noted that some transcripts are being spliced only once they diffuse away from the transcription site. These pre-mRNAs are easily identified as they retain their introns, and thus their intronic and exonic smFISH signals colocalize in multiple discrete fluorescent spots of similar size and brightness in the nucleoplasm away from transcription site or in the cytoplasm[32,33].

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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