

# Single-molecule mRNA detection and counting in mammalian tissue

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**We present a protocol for visualizing and quantifying single mRNA molecules in mammalian (mouse and human) tissues. In the approach described here, sets of about 50 short oligonucleotides, each labeled with a single fluorophore, are hybridized to target mRNAs in tissue sections. Each set binds to a single mRNA molecule and can be detected by fluorescence microscopy as a diffraction-limited spot. Tissue architecture is then assessed by counterstaining the sections with DNA dye (DAPI), and cell borders can be visualized with a dye-coupled antibody. Spots are detected automatically with custom-made software, which we make freely available. The mRNA molecules thus detected are assigned to single cells within a tissue semiautomatically by using a graphical user interface developed in our laboratory. In this protocol, we describe an example of quantitative analysis of mRNA levels and localization in mouse small intestine. The procedure (from tissue dissection to obtaining data sets) takes 3 d. Data analysis will require an additional 3–7 d, depending on the type of analysis.**

## INTRODUCTION

### Development of the protocol

Single-molecule RNA fluorescence *in situ* hybridization (smFISH) was first demonstrated by Femino *et al.*<sup>1</sup>, who used several oligonucleotide probes, labeled with five or six fluorophores, to visualize individual actin mRNA molecules. A slightly different method was developed by Raj *et al.*<sup>2</sup> in an elegant work describing single mRNA detection in yeast, *Caenorhabditis elegans*, fruit fly and mammalian cell lines. In this method, multiple (50–100), 20-base-long oligonucleotides, which are complementary in tandem to an mRNA sequence of interest (and each of which is labeled with a single fluorophore), collectively bind to the target RNA molecule. This interaction results in the appearance of a diffraction-limited spot, which is the smallest spot that can be observed by optical microscopy. The number of such spots corresponds to the number of target molecules. Detecting and counting these spots can be fully automated—an approach that provides a powerful tool for determining the absolute mRNA copy numbers in individual cells.

This protocol is a modification of the previously described smFISH method<sup>2</sup>, and it enables researchers to apply smFISH to histological samples (that is, tissue sections)<sup>3,4</sup>. This modification makes possible the simultaneous detection of several mRNA species (including those expressed at very low levels) in single cells within intact histological tissue sections. Importantly, no genetic modification (reporter expression, molecular tagging) is necessary to visualize and quantify the expression of genes of interest. Therefore, the range of target tissues in which mRNAs can be detected expands to include samples from humans.

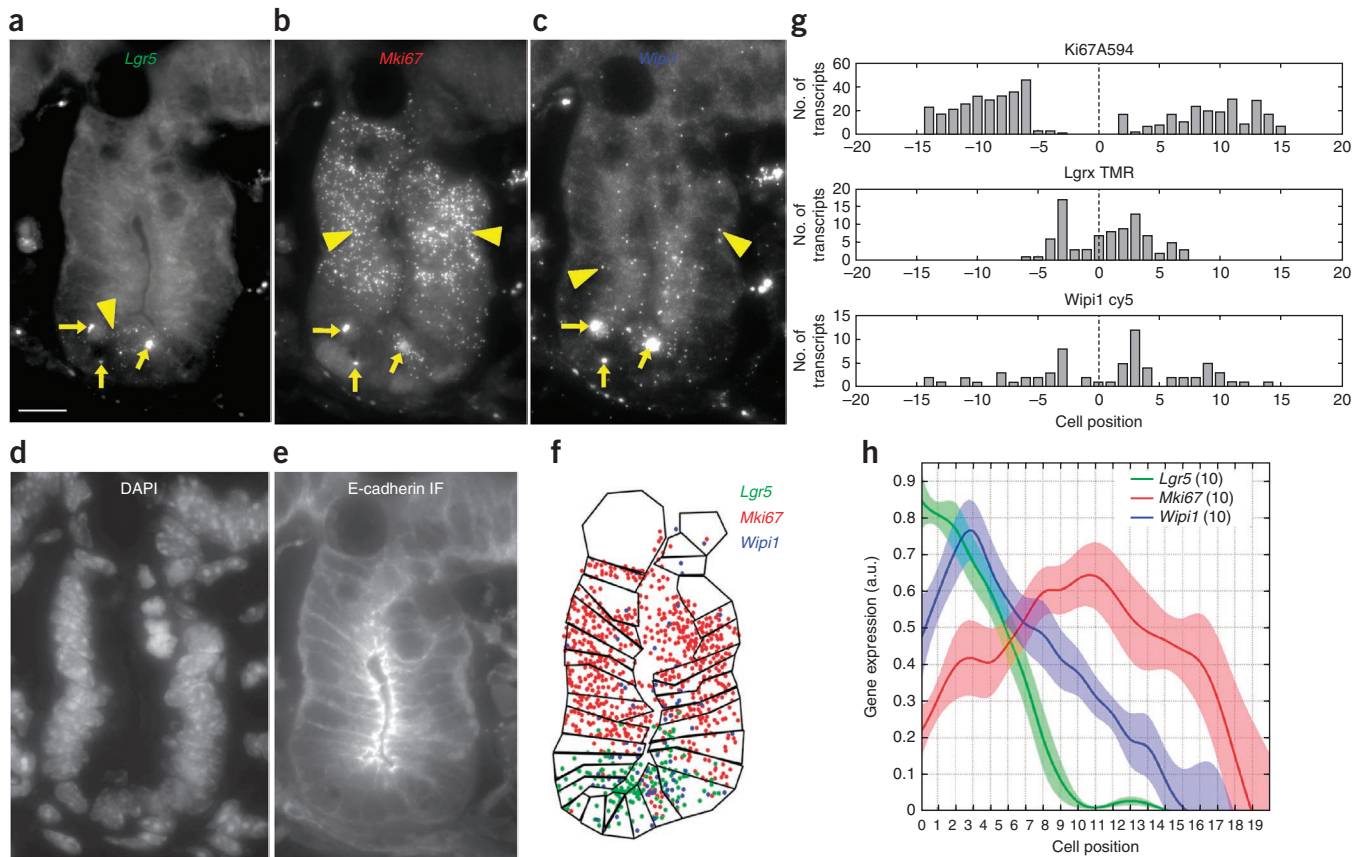
Our protocol is based on and similar to the method described in Raj *et al.*<sup>2</sup>. However, it involves additional fixation steps that are crucial to preserving RNA integrity in a variety of tissues, which often contain RNases. A few more additions to the original protocol, such as tissue cryopreservation, preparation of glass coverslips,

cryosectioning parameters and several others, are important for generating high-quality tissue samples and for obtaining a strong, quantifiable smFISH signal. Finally, we have generated custom software, called ImageM, which we provide upon request and which is designed to determine mRNA copy numbers in individual cells in histological organ tissue sections. This software is based on an existing algorithm that detects the smFISH signal (diffraction-limited fluorescence spots), assigns the spots to single cells and enables users to create expression maps for multiple mRNA species in the tissue of interest (in our case, in the intestine<sup>3</sup>). We have developed ImageM, a MATLAB-based graphical user interface (GUI) that makes image data analysis straightforward and user friendly; we make it freely available to readers upon request.

### Applications of the method

This protocol enables researchers to determine the absolute numbers of mRNA molecules in single cells within a tissue. The absolute numbers obtained by our technique are in a close correspondence to those measured by different means, for instance, via quantitative reverse-transcription PCR (qRT-PCR)<sup>1,2</sup>. As few as one mRNA molecule per cell can be detected. The maximum number of individual molecules that can be resolved microscopically is of the order of several thousand. This upper limit is dictated by the ratio between the cell volume and the typical voxel taken by a diffraction-limited mRNA dot ( $\sim 0.2 \times 0.2 \times 0.3 \mu\text{m}$ ).

Three different mRNA species can be simultaneously visualized (using probe libraries differentially labeled with Cy5, Alexa Fluor 594 and tetramethylrhodamine (TMR)) and quantified in parallel by implementing the present protocol; this upper limit is determined by the number of different fluorophores that can be spectrally resolved. Simultaneous staining with a DNA-binding dye and an antibody against cell-cell adhesion proteins enables the visualization of cell nuclei and borders,



**Figure 1** | Single-molecule RNA detection and quantification in mouse small intestinal cryosections. (**a–c**) Spots corresponding to single mRNA molecules resulting from the transcription of the gene *Lgr5* (**a**), detected with the TMR-labeled oligonucleotide probe library; the gene *Mki67* (also known as *Ki67*) (**b**), detected with the Alexa Fluor 594-labeled oligonucleotide probe library; and the gene *Wipi1* (**c**), detected with the Cy5 probe library. (**d**) DAPI nuclear staining. (**e**) Lateral membrane staining with a FITC-conjugated antibody specific to E-cadherin. Scale bar, 10  $\mu$ m. (**f**) Automatic quantification of spots in channels **a–c** and manual segmentation of cells on the basis of DAPI and E-cadherin immunofluorescence. Quantification based on 15 optical sections spaced 0.3  $\mu$ m apart in the z-direction. (**g**) Histogram of the numbers of mRNA molecules per cell for the three measured genes. Position 0 is the crypt apex. (**h**) Cryptogram of the genes in **a–c** on the basis of 10 analyzed crypts. Shown is the averaged spatial gene expression profile. Cell position here is the cell number above the crypt apex. Gene expression is the number of transcripts per cell divided by the maximum expression per crypt. Please note that in **a–c**, arrows denote nonspecific fluorescent objects and arrowheads indicate diffraction-limited spots representing single mRNA molecules. IF, immunofluorescence.

which is a requirement when the aims are to assign mRNA spots to cells within intact tissue.

As an example of quantitative and spatial analysis of gene expression, here we use the crypt of Lieberkühn, a functional unit of the intestinal epithelium, as a target tissue. First, the numbers of diffraction-limited spots are automatically detected using algorithms previously developed in our laboratory<sup>2,3</sup>. Next, cells are manually segmented on the basis of DAPI nuclear staining and E-cadherin lateral membrane staining, and spots are then assigned to individual cells. The resulting single-cell gene-expression profiles can be presented as a spatial profile (Fig. 1), which we termed ‘cryptogram’, from ‘crypt’ and ‘histogram’. This approach enables the experimenter to assess the precise spatial location of the expression of genes of interest in multiple crypts in the tissue of interest<sup>3</sup> (Fig. 1h).

In addition to the intestine<sup>3–6</sup>, the three-color smFISH protocol can be applied to a variety of tissues, including mammary glands<sup>7</sup>, kidney<sup>8</sup>, stomach, lymph nodes, mouse embryos at different gestational stages, human breast tumors<sup>9</sup> and possibly many more. Although the quality of FISH signal is consistently high in the different tissues tested, autofluorescence and/or

nonspecific probe binding may emerge in some tissues, and it may need to be addressed if it interferes with spot detection and/or data analysis. Examples of tissues that we have found to be particularly high in autofluorescence are skin (especially hair shafts and cornified layers of epidermis; A.L., unpublished observation), stomach (parietal cells contain highly autofluorescent cytoplasmic vesicles; A.L., unpublished observation) and small intestine (Paneth cells with highly autofluorescent cytoplasmic granules<sup>3</sup>). smFISH can also be combined with other techniques, such as EdU staining<sup>4</sup>, immunofluorescence<sup>4</sup> and DNA FISH<sup>9</sup> for probing single cells in intact tissues.

Given that hybridization conditions for different probe sets are similar, this method has great potential for the simultaneous detection of several mRNA species. As mentioned, the number of mRNA species to be detected simultaneously is limited by the availability of fluorophores that can be spectrally resolved using current technology. We routinely detect three different transcripts at the same time, using Cy5, Alexa Fluor 594 and TMR fluorophores. Other fluorophores emitting light at different wavelengths can be used as well, thus increasing the number of mRNAs that can be simultaneously detected<sup>10</sup>. Furthermore, combinatorial

labeling, in which different probe subsets are coupled to different fluorophores, can further increase the number of different color combinations that can be computationally resolved<sup>10,11</sup>. This approach may enable truly multiplexed single-molecule mRNA imaging, which would greatly facilitate large-scale gene expression profiling in single cells, in the context of tissues.

Cell border detection (segmentation) may become a challenge in tissues for which no good antibody against cell membrane-associated proteins is available. In these cases, cell borders may be visualized by other means, such as by using fluorescently labeled phalloidin or GFP-fusion membrane-associated proteins. An example of the latter is the use of tissues from a knock-in mouse expressing E-cadherin fused to cyan fluorescent protein (CFP)<sup>12</sup>. Alternatively, when cell border labeling is impossible, too laborious or not crucial, tissue surface can be divided into arbitrary (virtual) sectors ('cell proxies') and mRNA spots can be assigned to these proxies instead of cells. After the segmentation step, spatial analysis of mRNA distributions can be performed according to the topological features of the tissue.

In addition to tissue sections, our protocol can be applied to whole-mount organs or embryos with no substantial modifications. The only challenging part of such an application is imaging depth, as the smFISH signal quality from parts lying deeper than 10  $\mu\text{m}$  below the objective will decrease substantially. This problem can be alleviated by using confocal microscopy. The best results so far are obtained on a spinning-disk microscope (quantifiable signal from tissue layers 30–50  $\mu\text{m}$  deep tissue).

### Comparison with other methods

Overall, very few methods enable the detection of mRNA in single cells in the context of tissues<sup>13</sup>. Conventional *in situ* hybridization (ISH) enables the visualization of mRNA species present in tissue samples (sections or whole mount)<sup>14</sup>. For signal amplification and visualization, this method uses enzymatic color reactions. Dye particles are generated at the location of the probe and can then diffuse away from this site. As a consequence, ISH spatial resolution is lower than that of smFISH.

Unlike in conventional ISH and FISH, in our method, probe preparation does not include cloning steps, and it takes minimal effort. Notably, probes against different mRNA species work at standardized conditions, making them highly compatible with each other in a multicolor hybridization, which is often not the case in traditional ISH and FISH.

Finally, the conventional ISH protocol takes more than 3 d to complete and includes substantial sample digestion, which facilitates probe penetration into the tissue but also affects tissue integrity. The protocol described here can be completed within 3 d, from the dissection of the tissue to collecting data (imaging). It includes only one optional, very mild proteinase K digestion step, as the oligonucleotide probes are short (20 bp) and readily penetrate the sample after ethanol permeabilization. Samples need to undergo digestion only when suboptimal signal quality needs to be improved.

Although the absolute mRNA copy numbers assessed by our method are comparable to those obtained by qPCR<sup>2</sup>, the latter method is usually only applied to cell populations out of the context of a tissue. Laser-capture microdissection followed by RNA extraction and analyses addresses this issue<sup>15</sup>. However, given the small amounts of RNA obtained by this approach, the analyses

require substantial amplification of the material, which inevitably introduces inaccuracy in measurements. By contrast, smFISH enables the direct measurement of mRNA copy numbers without the need for amplification.

Rolling-circle amplification of padlock probes<sup>16</sup> is an additional technique that has demonstrated detection of individual mRNA in intact mouse embryonic tissue. However, the technique requires reverse-transcription and amplification steps, which are not required by smFISH.

Of note, the number of mRNA species that can be quantified by smFISH is relatively limited compared with the genome-wide scale of analysis, which is possible using extracted RNA via the new generation of gene expression analysis strategies, such as RNA-seq<sup>17,18</sup>. Furthermore, RNA-seq-based analysis can now be performed at a single-cell level<sup>19</sup>. However, the main advantage of smFISH is its ability to quantify mRNA levels in individual cells without taking them out of their biological environment. As for the mRNA species number limitation, recently developed methods have expanded the upper limit of transcripts that can be detected using smFISH in a single cell by clever multiplexing technology to up to 32 (refs. 10,20).

### Limitations

The first important concern when implementing smFISH is RNA integrity in the tissue sample. It is important that the tissue be dissected and fixed as quickly as possible and kept at a low temperature at all times. This requirement can become an issue when clinical samples are being harvested, as this is a task that is often not performed or supervised by people familiar with the protocol.

Another limitation is the size of the target mRNA. As mentioned above, to achieve sufficient signal strength, the probe library should consist of at least 20 probes, but the signal-to-noise ratio increases with more probes, and 48 probes are recommended as a default in the present protocol. A number of oligonucleotide probes this high is, however, often unachievable when probing for the presence of short mRNAs. In such cases, the signal-to-noise ratio is often not high enough for appropriate analysis.

As many tissues contain sources of autofluorescence, having to use small probe sets with low signal-to-noise ratios may become a problem. In these cases, the protocol may need modification to reduce autofluorescence.

Although nonspecific binding is less of a problem in smFISH than in antibody staining, it does exist mainly in tissue samples. Glandular tissues, such as stomach or intestine, are especially difficult to study, as certain cell types absorb all probes nonspecifically, which results in strong staining and masks the real signal. Paneth cells in the small intestine serve as an example. Multiple cytoplasmic granules, typical for these cells, often absorb probes nonspecifically, which gives rise to large aggregates of fluorescent matter appearing within Paneth cells (Fig. 1a–c). Fortunately, the nonspecific staining is easily distinguished from the smFISH signal, as it often consists of spots that are not diffraction-limited but rather larger, and it mostly appears in all three fluorescence channels. In most cases, these nonspecific foci are detected by our software and filtered out, and thus they do not interfere with the analysis. However, in a few cases, nonspecific binding may require some serious consideration and troubleshooting.



## **Experimental design**

Oligonucleotide libraries are designed in agreement with the set of requirements established previously<sup>2</sup>, and then purchased. By the term 'library', we mean a set of oligonucleotides, each of which hybridizes to the same mRNA molecule. The 'collective' binding of singly labeled oligonucleotides to the target mRNA molecule results in the appearance of a single bright fluorescent spot. A typical library consists of 48 oligonucleotides, each 20 bp long. The number of oligonucleotides in a library should exceed 20 (ref. 2), and it often is as large as 96.

The minimum size of a library depends on the length of the target mRNA. This target molecule should be sufficiently long to accommodate at least 20 oligonucleotides, plus a two-base space between neighboring oligonucleotides. Other important parameters are the G-C content of the library, which should be as close to 45% as possible, and the uniqueness of the target sequence (to minimize binding of the probes to anything other than the target mRNA). Conveniently, the 'Probe Designer' tool (freely available at <http://www.biosearchtech.com/stellarisdesigner/>) will guide a user through probe design, taking all the important parameters into consideration.

The 3'-end of each library oligonucleotide is coupled to a single fluorophore (Steps 1–5), and the resulting labeled oligonucleotide is purified (Steps 6–10). Alternatively, the sets of fluorophore-coupled and purified oligonucleotides can be purchased from Biosearch Technologies under the brand name Stellaris, or from another company of choice.

The tissue is dissected right after euthanizing the animal, and it is immediately fixed (Steps 12–16). After a cryoprotection step (Step 16), frozen blocks are prepared (Steps 17–20). Cryosections of 5–10- $\mu$ m thickness are mounted onto cleaned

and poly-L-lysine-coated glass coverslips (Steps 21–26). The attached sections are fixed again, rinsed and permeabilized (Steps 24–26), and then rehydrated and hybridized with the probes and antibody overnight (Steps 30–32). An antibody can be used to stain the cell borders, so as to visualize tissue structure. However, if hybridization conditions are not optimal for staining with the necessary antibody, alternative counterstaining methods should be considered, such as staining with fluorescently labeled phalloidin (Step 35). After hybridization, sections on coverslips are washed, counterstained with nuclear dye and equilibrated with the imaging buffer. If phalloidin staining is chosen to visualize the cell border, this staining is performed at this point, and sections are subsequently rinsed and mounted (Steps 33–36).

Images are acquired using an inverted epifluorescence microscope equipped with an automated stage, a cooled back-illuminated charge-coupled device (CCD) camera and a  $\times 100$  oil-immersion objective with a numerical aperture of 1.4 (Steps 37–39). At each position, a 5–10- $\mu$ m-thick z-stack of images with 0.2–0.4  $\mu$ m of distance between individual planes is acquired in order to collect the information from the whole thickness of the section.

The data are analyzed using MATLAB software (Steps 40–42). This software enables the automatic detection of the mRNA molecule spots, assists in manually segmenting cell borders and assigns the spots to individual cells, labeled according to position within the tissue relative to a reference (for instance, the bottom of an intestinal crypt). All automated and manual steps are integrated into ImageM, a straightforward and user-friendly GUI developed in our lab (**Box 1** and **Figs. 2** and **3**).

Key analysis of the gene expression signatures created can include spatial mapping of gene expression, single-cell gene expression profiles and single-cell correlations of pairs of genes<sup>3</sup>. In intestinal

## **Box 1 | Guide to using ImageM, a GUI facilitating smFISH data analysis**

**▲ CRITICAL** To open files, several options are available. For new analyses, use 'Open' (Ctrl + O) option to open .tiff image files. To open existing analyses, use 'Open Saved Result' (Ctrl + N) option to open .ima data files. To save files, use 'Save' (Ctrl + S) or 'Save as' options at any time to save the working data set in MATLAB data format with .ima suffix. The current figure window can be saved as an image file by using 'Export as Figure'.

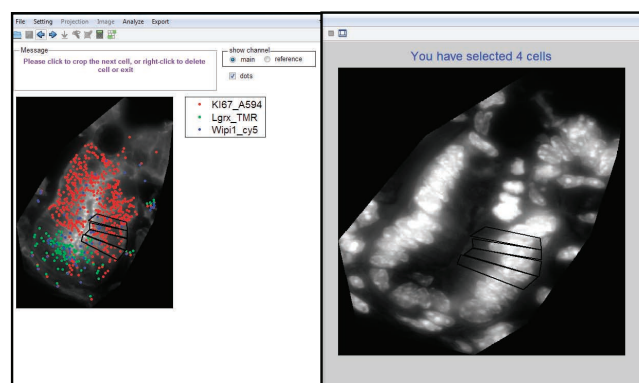
1. Open .tiff images. E-Cadherin channel is recommended for the crypt data. Please note that the message window displays the current status of the GUI.
2. Use 'Previous Stack' and 'Next Stack' to browse different stacks of the images so that only the focus stacks will be used for the following analysis.
3. Go to the 'Setting' menu, select 'Stack Filter' and then type the selected z optical sections that you would like to analyze in the 'From' and 'To' fields. Choose, typically, 5–12 z-positions (1.5–4  $\mu$ m in depth) and ensure that the positions do not have more than a single cell per z-stack.
4. Go to the 'Project' menu, select any options to conduct z-projection with the selected stacks of images.
5. Click the 'Enhance Image' button to improve image visualization. Alternatively, different options of image enhancement can be selected on the 'Setting' menu.
6. Click the 'Crop Image' button to select a region of interest. Double-click to proceed to the next step. Please note that at this juncture the GUI will prompt you to save the current analysis if you have not done so.
7. Click the 'Count Dots' button to start counting mRNA transcripts (dots). The GUI will prompt you to select the channels for this procedure.
8. For each channel, a new window will pop up. The GUI automatically preselects a threshold value for dot selection (**Fig. 3**). However, you will be able to manually modify the threshold value by clicking the desired value in the upper left figure. Click any key to proceed.
9. Click the 'Segment Cells' button to start segmenting cells. The GUI will prompt you to select the channels for this procedure. The DAPI channel is recommended as a reference for the crypt data. In addition, the main GUI window can switch between the main channel and the reference window. The dots and cells can be shown or hidden. To segment cells in crypts, segment one cell per longitudinal cross-section, such that cell position above the crypt apex will be well defined.

(continued)

## Box 1 | (Continued)

10. Left-click the mouse button in the image window to activate the area selection (for cell segmentation), and double-click to terminate the ongoing area selection. Right-click the mouse button within a segmented cell to either delete this segmented cell or stop segmentation. Please remember to stop segmentation after segmentation has been completed.
11. Click 'Reset cell labels' in the 'Analyze' menu to label the cells in a particular order. Use 'by click order' and click on the cells sequentially, starting at one end of the crypt outline and ending at the opposite end.
12. Go to the 'Analysis' menu and select 'detect background dots' to remove false-positive mRNA transcripts. This action removes dots that appear at the same spatial positions in multiple channels.
13. Go to the 'Analysis' menu and select 'Plot cell dot counts' to visualize basic data statistics.
14. Go to the 'Analysis' menu and select 'Plot Cryptogram--Unfolded' to create a cryptogram. You will be asked to draw out a rough crypt outline and point out the crypt apex. The GUI will prompt you to type in the threshold value (the maximum distance in pixels within which a dot will be allowed to be projected on the crypt outline) and the window size (for plotting). Note that this option plots the distribution of the number of transcript along the marked outline. A cell-based cryptogram similar to that shown in **Figure 1h** is performed by software that is not included in ImageM.
15. (Optional) All relevant variables of the segmented image are kept in the MATLAB structure array 'UserData', and the 'Initialization' function in the `imagem.m` file contains brief descriptions about the variables in 'UserData'. To access these descriptions, load the `.ima` by typing `load([dir file_name], '-mat')` in the command line. Alternatively, go to the 'Debug' menu, and select 'save to workspace'. A MATLAB data file name 'matlab.mat' will be saved to the working directory.

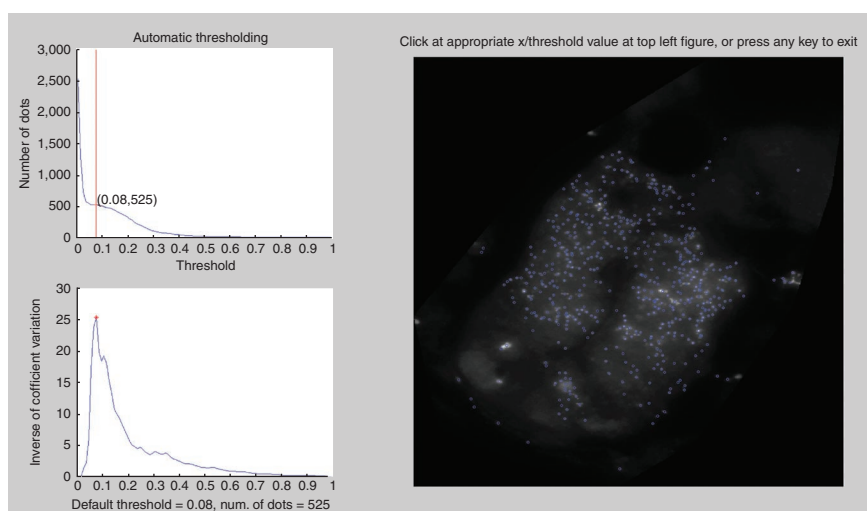
**Figure 2** | Screenshot of the workspace in the image analysis GUI ImageM, taken during cell segmentation. Left, action menu with shortcut buttons. Shown is an image of a crypt with single mRNA dots, detected by the software. The three colors, red, green and blue, denote three different mRNA species, translated from genes *Mki67* (also known as *Ki67*), *Lgr5* and *Wipi1*, respectively. Black contours are cell borders, manually drawn after E-cadherin antibody cell border staining. Right, the same crypt, stained with DAPI (labeling nuclei). This image is used as a reference for cell segmentation (the black contours created by the user on the E-cadherin image simultaneously appear on the DAPI image).



crypts, the position of cells above the crypt apex defines a natural one-dimensional axis. For each gene, a spatial gene expression profile can be created. Pooling profiles from different analyzed crypts enables one to obtain the average gene expression profile and its variance, which we term a cryptogram (**Fig. 1g,h**). Such an analysis is particularly suited to genes broadly expressed in a consistent manner between crypts. Separate analysis can be applied to other genes that show a more variable expression pattern in terms of the position of the expressing cells along the crypt axis.

In the mouse intestine, some genes are highly specific to particular differentiated cell types, which can appear in variable

positions in different crypts. Examples include markers for Goblet cells (*Clca3*) and Tuft cells (*Dclk1*, also known as *Dcamk1l*)<sup>3</sup>. An additional analysis, which can be performed using smFISH on tissue sections, is the characterization of single-cell gene expression signatures for such rarer cell types. The numbers of transcripts of other genes in such cells, defined by a marker of interest, can be compared to cells, which are directly above or below the cell of interest. This analysis stringently tests the upregulation or downregulation of particular genes in the cell type of interest, while controlling the spatial pattern of gene expression of the tested genes<sup>3</sup>. In addition, the Pearson or Spearman correlation coefficients of



**Figure 3** | Screenshot of the ImageM workspace, taken during the process of automated detection of diffraction-limited spots. Left, plots showing parameters of automatic spot detection. The red line indicates the threshold selected. Right, an image of smFISH with software-detected dots (blue circles). The user can manually correct the automatically selected threshold by clicking on the left plots.

the single-cell gene expression, correlated transcription patterns of pairs of genes may indicate the presence of common regulatory modules for these genes.

More advanced and sophisticated analyses can be performed on the resulting data sets. MATLAB codes are available upon request (see MATERIALS).

Finally, please note that although positive and/or negative controls for the expression of specific mRNAs are not covered in the procedure detailed below their use is likely to greatly improve the reliability of the protocol's results. A more in-depth discussion on this subject is reported in the ANTICIPATED RESULTS.

## MATERIALS

### REAGENTS

- Animals. Tissue samples can be collected from a broad spectrum of animals, from *Drosophila melanogaster* to mammals. **CAUTION** Researchers should follow appropriate procedural regulations when performing animal experiments. These regulations may vary depending on the legislation of the country in which the research is being conducted. Commonly accepted guidelines, such as the ARRIVE<sup>21</sup> guidelines, should be followed.
- DNA oligonucleotide probe set, with 3'-amine modification or coupled to a fluorophore (Biosearch Technologies, <http://www.singlemoleculefish.com>, or another company of your choice). Sequences of the three mRNAs detected in an example experiment (mRNAs for *Ki67*, *Lgr5* and *Wip1*) and the three corresponding probe libraries are listed in **Supplementary Table 1**
- 6-Carboxytetramethylrhodamine succinimidyl ester (TMR; Molecular Probes/Invitrogen, cat. no. C6123)
- Cy5 succinimidyl ester (GE Healthcare, cat. no. PA25001)
- Alexa Fluor 594 carboxylic acid succinimidyl ester (Molecular probes/Invitrogen, cat. no. A37572)
- FITC mouse anti-E-cadherin antibody (BD Transduction Laboratories, cat. no. 612130)
- Alexa Fluor 488 phalloidin (Life Technologies, cat. no. A12379; optional, see Experimental design)
- Sodium bicarbonate (Sigma-Aldrich, cat. no. S5761)
- DMSO (Sigma-Aldrich, cat. no. D1435)
- Triethylammonium acetate buffer, 1 M (Sigma-Aldrich, cat. no. 90358)
- Acetonitrile (Sigma-Aldrich, cat. no. 34998)
- Sodium acetate, pH 5.5, 3 M (Ambion, cat. no. AM9740)
- Tris, 1 M, pH 8.0 (Ambion, cat. no. AM9855G)
- Ethanol, absolute (J.T. Baker, cat. no. 8025) **CAUTION** Ethanol is flammable.
- Tris-EDTA, pH 8.0 (TE; Ambion, cat. no. AM9849)
- PBS, pH 7.4, RNase-free, 10× (Ambion, cat. no. AM9625)
- SSC, RNase-free, 20× (Ambion, cat. no. AM9763)
- Nuclease-free water (Ambion, cat. no. AM9932) or water filtered using a Milli-Q filtering system (Millipore, cat. no. CDUFBI001)
- Formamide, deionized, nuclease-free (Ambion, cat. no. AM9342)
- **CAUTION** Formamide is a toxic material and a teratogen. Handle it inside a fume hood; wear protective gloves and a lab coat.
- *Escherichia coli* tRNA (Roche, cat. no. 10109550001)
- BSA, nuclease-free, 50 mg ml<sup>-1</sup> (Ambion, cat. no. AM2616)
- Dextran sulfate, sodium salt (Sigma-Aldrich, cat. no. D8906)
- Ribonucleoside vanadyl complex, 200 mM (RVC; New England Biolabs, cat. no. S1402S)
- Formaldehyde, 37% (wt/vol) (Sigma-Aldrich, cat. no. F15587)
- **CAUTION** Formaldehyde is a toxic material and a carcinogen. Handle it inside a fume hood; wear protective gloves and a lab coat.
- Paraformaldehyde, 16% (wt/vol) (Electron Microscopy Sciences, cat. no. 15710) **CAUTION** Paraformaldehyde is a toxic material and a carcinogen. Handle it inside a fume hood; wear protective gloves and a lab coat.
- Proteinase K solution, 20 mg ml<sup>-1</sup> (Ambion, cat. no. AM2546)
- 4',6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich, cat. no. D9542)
- Poly-L-lysine, 0.1% (wt/vol) (Sigma-Aldrich, cat. no. P8920)
- Jung tissue freezing medium (Leica Microsystems) or OCT compound (TissueTek)
- Sucrose (Sigma-Aldrich, cat. no. S7903)
- Glucose (Sigma-Aldrich, cat. no. G8270)
- Glucose oxidase (Sigma-Aldrich, cat. no. G2133)
- Catalase (Sigma-Aldrich, cat. no. C3515)

- Trolox (Sigma-Aldrich, cat. no. 238813)
- RBS-35 (Fluka, cat. no. 83461)
- Rubber cement (Fixo Gum, cat. no. 290117000)
- Dry ice
- TetraSpeck microspheres, 0.2 μm (Invitrogen, cat. no. T-7280)
- Sodium hydroxide, 5 N
- Methanol

### EQUIPMENT

- Microcentrifuge tubes, 1.8 mg
- Six-well plates (BD Biosciences, cat. no. 351146)
- Petri dishes (BD Biosciences, cat. no. 351029)
- Parafilm (Sigma-Aldrich, cat. no. P7793-1EA)
- Sharp-tip forceps (ROBOZ, cat. no. RS-5095)
- Filter paper (Schleicher & Schuell, cat. no. 10310992)
- Speed vacuum concentrator (Eppendorf Concentrator Plus)
- Tabletop centrifuge (Eppendorf, cat. no. 5424R)
- Vortex Genie (Scientific Industries)
- NanoDrop 2000 spectrophotometer (Thermo Scientific)
- Falcon tubes, 15 mg (BD Biosciences, cat. no. 352196)
- Falcon tubes, 50 mg (BD Biosciences, cat. no. 352070)
- Tube rotator (Stuart, cat. no. SB2)
- Nutating mixer (VWR)
- Centrifuge (Eppendorf, cat. no. 5810R)
- RNase-free, low-retention pipette tips (GeneMate, cat. no. P-1237)
- Sonicator (VWR, Ultrasonic Cleaner)
- Desiccator
- Plastic molds for tissue embedding, cat. no. 6254-10 (10 × 10 × 5 mm); cat. no. 6254-15 (15 × 15 × 5 mm); cat. no. 6254-25 (25 × 20 × 5 mm) (frozen blocks; Tissue Tek Cryomold, Electron Microscopy Sciences)
- Cryostat (Cryostar NX70, Thermo Scientific)
- HPLC (Gilson GX271 liquid handler, with C18 Targa column and UV-visible 155 spectrometer, Higgins Analytical)
- Pre-column frit (Chrom Tech, cat. no. A-332X)
- Hybridization oven (UVP, HB-1000 hybridizer)
- Microscope glass slides (Thermo Scientific, Gold Seal, cat. no. 3031-002)
- Microscope cover glass, 22 × 22 mm, no. 1 (Thermo Scientific, cat. no. D10143263NR1)
- Sterile syringe filters, 0.2 μm (Millipore, cat. no. 6900-2502)
- Cover glass staining rack (Electron Microscopy Sciences, cat. no. 72241-01)
- Cover glass wash jar (Electron Microscopy Sciences, cat. no. 70312-23)
- Inverted fluorescence microscope (Nikon, Eclipse-Ti)
- Numerical aperture (NA) 1.4 objective, ×100 (Nikon)
- Immersion oil
- Metal halide lamp (Prior Lumen 220)
- High sensitivity CCD camera (Photometrics Pixis 1024B)
- Motorized optical shutter (Sutter SMARTSHUTTER, Sutter Instruments)
- Motorized stage controller (TI-S-E motorized stage)
- Filter cube for Cy5.5 (Chroma Technology, cat. no. 41023)
- Custom filter cube for Alexa Fluor 594: Omega Optical (exciter: cat. no. 2017901-590DF10, dichroic filter: cat. no. XF2014-610DRLP and emitter: cat. no. XF3028-630DF30)
- Filter cube for TMR (Omega Optical, cat. no. XF204)
- Computer of minimum 4 GB RAM
- Microscope managing and imaging software (Metamorph, Molecular Devices)
- MATLAB (The MathWorks)
- KNF Teflon pump



- Vacuum pump
- Styrofoam boxes with lids
- ImageM and other MATLAB codes developed in our lab are available upon request (requests can be addressed to S.I. ([shalev.itzkovitz@weizmann.ac.il](mailto:shalev.itzkovitz@weizmann.ac.il)), J.P.J. ([j.junker@hubrecht.eu](mailto:j.junker@hubrecht.eu)) and A.v.O. ([a.oudenaarden@hubrecht.eu](mailto:a.oudenaarden@hubrecht.eu)))

## REAGENT SETUP

**Definition of the target sequence** Choose a region of the target mRNA that is sufficiently long so that at least 20 probes—but preferably 48—each 20 bp long, can anneal to it, with at least two bases present between neighboring probe-binding sites (which amounts to at least 1,054 bp). If possible, avoid using untranslated regions (UTRs). **▲ CRITICAL** The presence of multiple binding sites for some of the probes in the library (other than the mRNA of interest) will lead to a high fluorescent background, which will decrease the quality of the FISH signal and may cause problems in spot detection and data analysis. If possible, exclude tandem repeats, microsatellites and other repeating sequences from the target sequence.

**Design of the library of probe oligonucleotides** Use the Probe Designer tool (<http://www.biosearchtech.com/stellarisdesigner/>) developed by Arjun Raj for this task. This tool is freely available, and no downloads are required; users only need to create their own login name and password, and then they can start using the Probe Designer immediately and free of charge. Follow stepwise instructions to upload the target sequence, to enter the information on the origin of the sequence, to specify the masking level and to create the list of oligonucleotide probes. Order the oligonucleotides on the list from Biosearch Technologies or another company of your choice. Please make sure to order sufficient quantities of the oligonucleotides in the most convenient format. In this protocol (probe-fluorophore coupling procedure, Steps 1–4), we use 10  $\mu$ l of each oligonucleotide at the concentration of 100  $\mu$ M (in water) per labeling reaction. In general, DNA oligonucleotides dissolved in water are stable for several years if they are stored below  $-20^{\circ}\text{C}$ . Repeated freeze-thaw cycles do not detectably affect the performance of our libraries in this method. However, please consider the manufacturer's instructions for reconstitution and storage of the oligos.

**Sodium bicarbonate, 1 M, pH 8.0** In a 50-ml Falcon tube, dissolve 4.2 g of the powder in 40 ml of distilled or deionized, nuclease-free water. Adjust the pH to 8.0 with 5 N sodium hydroxide. Adjust the volume to 50 ml.

**! CAUTION** Sodium hydroxide is highly caustic. Handle it carefully; wear protective gloves and a lab coat. **▲ CRITICAL** prepare this solution fresh and discard it after 2 weeks. Alternatively, divide the solution into 5-ml portions and freeze them. These aliquots can be stored at  $-20^{\circ}\text{C}$  for several months.

**Sodium hydroxide, 5 N** Dissolve 10 g of sodium hydroxide pellets in 50 ml of distilled or deionized water. Add the pellets in small quantities to the water, allow them to dissolve, cool the solution down, and then add the next portion of pellets. This solution can be stored indefinitely at room temperature ( $20$ – $25^{\circ}\text{C}$ ). **! CAUTION** Sodium hydroxide is highly caustic. Handle it carefully and wear gloves and a lab coat. Sodium hydroxide pellets absorb water from the air; weigh them quickly using glass or plastic weighing cups (not weighing paper). Dissolving sodium hydroxide is highly exothermic; keep the tube on ice. Do not pour water onto the pellets, as it will boil and splash out of the tube.

**Sodium bicarbonate, 0.1 M, pH 8.0** Combine 5 ml of the 1 M sodium bicarbonate and 45 ml of distilled or deionized nuclease-free water.

**▲ CRITICAL** Freshly prepare this solution each time or make sure to use it within 1 week of preparation.

**HPLC buffer A** Mix 100 ml of 1 M triethylammonium acetate with 900 ml of deionized water. Freshly prepare the buffer every week and store it at room temperature. **! CAUTION** Triethylammonium acetate is volatile; handle it in a fume hood while wearing protective gloves.

**HPLC buffer B** HPLC buffer B is undiluted acetonitrile. **! CAUTION** This is an organic solvent; handle it in a fume hood while wearing protective gloves. **DAPI, 20 mg ml<sup>-1</sup>** Add 500  $\mu$ l of deionized, nuclease-free water to 10 mg of DAPI powder. Divide the solution into 20- to 50- $\mu$ l portions and store them at  $-20^{\circ}\text{C}$  for up to several years.

**DAPI, 100  $\mu$ g ml<sup>-1</sup>** Add 990  $\mu$ l of deionized, nuclease-free water to 10  $\mu$ l of 20 mg ml<sup>-1</sup> DAPI. Store the solution at  $4^{\circ}\text{C}$  and use it within a month.

**Formaldehyde, 3.7% (vol/vol)** For a 50-ml solution, combine 40 ml of distilled or deionized, nuclease-free water, 5 ml of 37% formaldehyde (wt/vol) and 5 ml of 10 $\times$  PBS. Freshly prepare the solution just before use;

alternatively, prepare it in advance, divide it into 25- to 50-ml portions and freeze the portions. Store formaldehyde at  $-20^{\circ}\text{C}$  for up to several months and thaw it just before use. **! CAUTION** Formaldehyde is highly toxic and carcinogenic. Handle it in a fume hood while wearing a lab coat and protective gloves.

**Paraformaldehyde, 4% (vol/vol)** Combine 10 ml of 16% (wt/vol) paraformaldehyde, 4 ml of 10 $\times$  PBS and 26 ml of deionized, nuclease-free water.

Freshly prepare the solution just before use or prepare it in advance, divide it into 25- to 50-ml portions and store the portions at  $-20^{\circ}\text{C}$  for up to several months. Thaw the solution in a  $37^{\circ}\text{C}$  water bath just before use.

**! CAUTION** Paraformaldehyde is highly toxic and carcinogenic. Handle it in a fume hood while wearing a lab coat and protective gloves.

**Cryoprotecting solution** Combine 5 ml of 10 $\times$  PBS, 5 ml of 37% (vol/vol) formaldehyde and 15 g of sucrose. Add distilled or deionized, nuclease-free water to a final volume of 50 ml. Incubate the solution with gentle agitation at room temperature to allow the sucrose crystals to dissolve. Freshly prepare this solution just before use or prepare it in advance, divide it into 25- to 50-ml portions and store the portions at  $-20^{\circ}\text{C}$  for up to several months. Thaw the solution in a  $37^{\circ}\text{C}$  water bath just before use.

**! CAUTION** Formaldehyde is highly toxic and carcinogenic. Handle it in a fume hood while wearing a lab coat and protective gloves.

**RBS-35, 2% (vol/vol)** Combine 20 ml of RBS and 980 ml of distilled or deionized water. Store the solution at room temperature for up to several months.

**Ethanol, 70% (vol/vol)** Combine 700 ml of absolute ethanol and 300 ml of distilled or deionized nuclease-free water. Store it indefinitely in a tightly closed container at room temperature.

**Poly-L-lysine** Combine 20 ml of 0.1% (wt/vol) poly-L-lysine solution and 180 ml of deionized or distilled, nuclease-free water. Freshly prepare the solution before use and discard the remaining solution after use.

**Ribonucleoside vanadyl complex** Incubate the purchased solution in a  $65^{\circ}\text{C}$  water bath for 10 min, divide it into 100- $\mu$ l aliquots and store them at  $-20^{\circ}\text{C}$  for up to several months.

**Glucose oxidase, 100 $\times$  stock** Dissolve the enzyme in 50 mM sodium acetate (pH 5.0), to a concentration of 3.7 mg ml<sup>-1</sup>. Divide the solution into 50- to 100- $\mu$ l aliquots and store them at  $-20^{\circ}\text{C}$  for up to several months.

**Trolox, 200 mM (100 $\times$  stock)** Dissolve 5 mg of the Trolox powder in 1 ml of pure ethanol. Divide the solution into 50- to 100- $\mu$ l portions and store them at  $-20^{\circ}\text{C}$  for up to several months.

**Glucose 10% (wt/vol)** Dissolve 5 g of glucose in 50 ml of deionized, nuclease-free water. Sterilize the solution by passing it through a 0.2- $\mu$ m syringe filter and store it at  $4^{\circ}\text{C}$  for up to a few weeks.

**GLOX buffer** Combine 100  $\mu$ l of Tris (1 M, pH 8.0), 1 ml of 20 $\times$  SSC, 400  $\mu$ l of 10% (wt/vol) glucose and 8.5 ml of deionized, nuclease-free water. This solution needs to be prepared freshly at least every week. For best results, prepare the solution just before use.

**GLOX buffer with Alexa Fluor 488 phalloidin** Reconstitute Alexa Fluor 488 phalloidin as described in the manufacturer's manual. Briefly, add 1.5 ml of methanol to 300 units of Alexa Fluor 488 phalloidin and dissolve it by passing it several times through a pipette tip. Add 4  $\mu$ l of the reconstituted Alexa Fluor 488 phalloidin to 2 ml of GLOX buffer. Freshly prepare this solution before use.

**Imaging buffer** Add 1  $\mu$ l of glucose oxidase (stock 3.7 mg ml<sup>-1</sup>), 1  $\mu$ l of catalase (vortex catalase suspension just before pipetting, do not spin) and 1  $\mu$ l of Trolox (200 mM stock) to 100  $\mu$ l of GLOX buffer. Use this solution immediately, although it may be stored on ice or at  $4^{\circ}\text{C}$  for up to 8 h.

**Formamide** Before opening the formamide bottle, bring it to room temperature. Divide the solution into 10- to 50-ml portions and store them in the dark at  $4^{\circ}\text{C}$  for up to several months. **! CAUTION** Formamide is toxic and teratogenic. Handle it in a fume hood while wearing protective gloves and a lab coat.

**Wash buffer, 10% (vol/vol) formamide** Combine 5 ml of 20 $\times$  SSC, 5 ml of formamide and 45 ml of deionized, nuclease-free water. Freshly prepare the solution before use. **! CAUTION** Formamide is toxic and teratogenic. Handle it in a fume hood while wearing protective gloves and a lab coat.

**Wash buffer with DAPI** Prepare the wash buffer, 10% (vol/vol) formamide, as described above. Add 50  $\mu$ l of DAPI (100  $\mu$ g ml<sup>-1</sup> stock) to 10 ml of wash buffer (1:200 dilution). Use this solution immediately. **! CAUTION** Formamide is toxic and teratogenic. Handle it in a fume hood while wearing protective gloves and a lab coat.

## PROTOCOL

**E. coli tRNA** Dissolve the powder in deionized, nuclease-free water to a concentration of 20 mg ml<sup>-1</sup>. Divide the solution into 500-μl portions and store them at -20 °C for up to several months.

**Hybridization buffer, 10% formamide** In a 15-ml conical Falcon tube, combine 7.3 ml of deionized, nuclease-free water, 1 ml of 20× SSC and 1 g of dextran sulfate. Vortex the mixture, and then incubate it on a tube rotator until the dextran is fully dissolved (~30 min). Add 1 ml of formamide, 500 μl of tRNA stock (20 mg ml<sup>-1</sup>), 100 μl of RVC stock (200 mM) and 40 μl of BSA stock (50 mg ml<sup>-1</sup>). Mix the buffer by inverting the tube; divide the solution into 300- to 500-μl portions and store them at -20 °C for up to several months. **! CAUTION** Formamide is toxic and teratogenic. Handle it in a fume hood while wearing protective gloves and a lab coat.

**Proteinase K working solution** Make 1:1,000–1:2,000 dilutions of the purchased stock in 2× SSC in nuclease-free water. Freshly prepare this solution before use.

### EQUIPMENT SETUP

**Cover glass preparation** Arrange cover glasses on the racks, one per slot, making sure they do not cling to each other. Place the racks into a wash jar. Add 200 ml (or enough to cover the glasses) of the 2% (vol/vol) RBS-35 to the jar, place the jar into a sonicator bath and sonicate it for 15 min. Pour off the 2% (vol/vol) RBS-35 and rinse the cover glasses with deionized or distilled water by filling the jar up and pouring the water out 7–10 times. Use nuclease-free water for the last rinse. Pour 200 ml of 70% (vol/vol) ethanol into the jar and repeat the 15-min sonication. Rinse the cover glasses once with nuclease-free water. Place the racks with clean dry glasses into a clean dry jar, and add 200 ml (or enough to

cover the glasses) of 0.01% (wt/vol) poly-L-lysine solution. Incubate the glasses at room temperature for 30–45 min. Take the racks out and air-dry the cover glasses. **▲ CRITICAL** Although the length and width of the cover glass to which tissue sections will attach may vary (22 × 22 mm cover glasses are preferred in our laboratory), the thickness should not exceed 0.15 mm (glass no. 1 or no. 0.5).

**▲ CRITICAL** Coated dried cover glasses should be used within 1 or 2 d, or they may be stored under desiccation at room temperature for several weeks.

**Sharp-tip forceps preparation** Wash the forceps with 2% (vol/vol) RBS-35 and rinse it with nuclease-free water. Air-dry the forceps.

**Cryo-sectioning settings** We normally set the cryostat chamber and specimen temperature to between -19 °C and -21 °C. These parameters may vary slightly depending on the histologists' preferences. Importantly, the sample must be kept frozen solid at all times. Section thickness is set between 5 and 10 μm. **▲ CRITICAL** Section thickness greater than 10 μm may cause an increase in background fluorescence during imaging.

**Microscope and imaging** We use an inverted epifluorescence microscope (Eclipse Ti, Nikon), fitted with a high-quantum-efficiency cooled CCD camera, motorized shutters, motorized stage, a metal halide lamp (Prior Lumen 220) as a light source and an ×100 high-NA oil-immersion objective. We use band-pass filter cubes optimized for imaging three separate fluorophores (Cy5, Alexa Fluor 594 and TMR) at the same time. Microscopy and imaging setup is controlled by Metamorph software (Molecular Devices). Other epifluorescence microscopes can be used as well. **▲ CRITICAL** A sensitive, cooled, back-illuminated CCD camera (e.g., Photometrics Pixis 1024B) and a ×100 magnification objective with a high NA (>1.3) are necessary for imaging.

## PROCEDURE

### Coupling FISH probe libraries to fluorophores ● TIMING 2–4 h

**1|** Pool the oligonucleotides belonging to one library (a set of oligonucleotides that can hybridize to a single mRNA molecule) in a 1.8-ml microcentrifuge tube (10 μl of each 100 μM solution per coupling reaction). The final solution volumes will vary depending on oligonucleotide library size. For the experiment described as a template in this protocol, the probe library against mouse *Lgr5* (**Supplementary Table 1**) consists of 96 oligonucleotides, and therefore the final volume will be 960 μl. The *Ki67* probe library is of the same size; therefore, the final volume will be the same, that is, 960 μl. By contrast, the *Wipi1* library consists of 48 oligonucleotides; therefore, the final volume of the pooled oligonucleotides will be 480 μl. As the solvent will be evaporated, the volume does not need to be adjusted. Mix the solution and dry it in a vacuum concentrator to complete evaporation. To reduce the drying time, split the oligonucleotide mixture into small portions (50–100 μl) and heat them up to 60 °C.

**2|** Resuspend each dried library in 70 μl of 0.1 M sodium bicarbonate, pH 8.0. In the experiment detailed here, prepare three tubes containing the libraries: the first against *Lgr5*, the second against *Ki67* and the third against *Wipi1*.

**▲ CRITICAL STEP** A pH value of 8.0–8.5 is a prerequisite for an efficient coupling.

**3|** Resuspend the dry Alexa Fluor 594 and Cy5 in 0.1 M sodium bicarbonate. When you reconstitute the dyes, consider that one vial of Alexa Fluor 594 (1 mg) contains enough dye for up to 10 coupling reactions (enough to label 10 separate, 48-oligonucleotide-sized libraries, with the same color), whereas one vial of Cy5 contains enough dye for up to 5 coupling reactions (i.e., sufficient to label up to 5 separate, 48-oligonucleotide-sized libraries, with the same color). The volume of 0.1 M sodium bicarbonate used for dye reconstitution depends on the numbers of libraries or oligos that have to be coupled to the same dye. The volume for labeling one 48-oligonucleotide library is 30 μl. For example, to couple five libraries of 48 oligos to Cy5, add 150 μl of buffer to the dry dye, resuspend it and use 30 μl per library reaction (each library in a separate tube). To couple ten separate 48-oligo libraries to Alexa Fluor 594, resuspend the dye in 300 μl of buffer and use 30 μl per reaction. To couple a larger library, the quantity of dye needs to be increased. For example, a 96-oligonucleotide library will need 60 μl of the dye solution. In the present experiment, *Ki67* library requires 60 μl of the Alexa Fluor 594 solution, and the *Wipi1* library requires 30 μl of the Cy5 solution.

**▲ CRITICAL STEP** Once they have dissolved, the dyes quickly lose reactivity, so please proceed to Step 5 of the PROCEDURE quickly. The reconstituted dye cannot be stored and used later; therefore, all unused dye must be discarded.

**4|** Dissolve 1–3 mg of TMR powder (measuring 1 mg of TMR powder is difficult; any amount between 1 and 3 mg will suffice) in 5 μl of DMSO. As with other dyes, add 30 μl of 0.1 M sodium bicarbonate per coupling reaction to the dye. For example, to couple three oligonucleotide libraries, add 90 μl to the dry dye, resuspend it and use 30 μl per reaction. In the present experiment, 60 μl of TMR solution is required to label the *Lgr5* library, which consists of 96 oligonucleotides.



5| Add 30  $\mu\text{l}$  of the dye solutions prepared in Steps 4 and 5 to each tube containing the pooled probe oligonucleotides (70  $\mu\text{l}$ , one library per tube). Mix Alexa Fluor 594 with the *Ki67* probe library mix, Cy5 with the *Wipi1* probe library mix and TMR with the *Lgr5* probe library mix. The final volume of the reaction will be 100–130  $\mu\text{l}$ . The final volumes of the coupling reactions for the probes for *Ki67* and *Lgr5* are 130  $\mu\text{l}$  each, whereas the *Wipi1* coupling reaction volume is 100  $\mu\text{l}$ . Mix and incubate the tubes at room temperature for 1–3 h, protecting the tubes from light (there is no need to agitate the tubes).

#### Probe precipitation, washing and purification ● TIMING 1–2 d

6| Add a 10–12% volume of 3 M sodium acetate (pH 5.5) and at least 2.5 volumes of 100% ethanol to each tube and mix the contents. In the experiment described, add 15  $\mu\text{l}$  of 3 M sodium acetate (pH 5.5) and 350  $\mu\text{l}$  of ethanol to each *Lgr5* and *Ki67* libraries' coupling reactions; add 12  $\mu\text{l}$  of 3 M sodium acetate (pH 5.5) and 300  $\mu\text{l}$  of ethanol to the *Wipi1* coupling reaction. Incubate the mixture at  $-80\text{ }^{\circ}\text{C}$  for at least 1.5 h and up to overnight.

7| Centrifuge the tubes at maximum speed in a tabletop centrifuge at  $4\text{ }^{\circ}\text{C}$  for 20 min.

8| Remove and discard the supernatant, and then wash the pellet with cold 70% (vol/vol) ethanol. Do not vortex. Centrifuge the mixture at maximum speed in a tabletop centrifuge at  $4\text{ }^{\circ}\text{C}$  for 10 min. Remove the supernatant carefully and completely. Air-dry the pellet.

■ **PAUSE POINT** The dried pellet can be stored for up to a year at  $-20\text{ }^{\circ}\text{C}$ .

9| HPLC-purify the fluorophore-labeled oligonucleotides. Various setups can be used for this purpose. We use a Gilson GX271 liquid handler with a C18 column and a UV-visible 155 spectrometer. We use a buffer A composed of 0.1 M triethylammonium acetate (pH 6.5), and a buffer B composed of acetonitrile (pH 6.5) for the gradient, which ranges from 7 to 23% buffer B in buffer A over the course of 45 min, followed by 80% buffer B for 5 min and another 15 min with 7% buffer B before running another sample.

▲ **CRITICAL STEP** Use a pre-column frit (A-332X) to prevent clogging.

10| Dry the samples in a vacuum concentrator, first by using a KNF Teflon pump for 2–5 h to remove acetonitrile, and then by drying overnight with a vacuum pump. Labeling efficiency routinely achieved is close to 90%. The resulting yield of labeled oligonucleotides is  $\sim 200\text{ }\mu\text{g}$ .

#### Probe reconstitution and storage ● TIMING 5–10 min

11| Reconstitute the labeled oligonucleotide library in 30–50  $\mu\text{l}$  of TE (pH 8.0). This probe stock ( $2\text{--}4\text{ }\mu\text{g }\mu\text{l}^{-1}$ ) is normally used at a final dilution of 1:1,000–1:5,000. It is convenient to make at this stage a 1:100 intermediate dilution (in TE buffer, pH 8.0). To determine which probe dilution will give the highest FISH signal intensity with the lowest background fluorescence, we recommend doing a coarse probe titration by adding 2–10  $\mu\text{l}$  of the intermediate dilution to 100  $\mu\text{l}$  of hybridization buffer (used for one  $22 \times 22\text{ mm}$  coverslip with tissue sample on it), to find an optimal dilution range for each probe library.

■ **PAUSE POINT** Both the stock and the 1:100 dilution can be stored at  $-20\text{ }^{\circ}\text{C}$  for several months. However, we do not recommend storing working dilutions.

#### Tissue dissection, fixing and cryoprotection ● TIMING 4 h to overnight

▲ **CRITICAL** Please note that consulting basic guides on mouse tissue pathology<sup>22</sup> (<http://ctrngenpath.net/static/atlas/mousehistology/>), dissection<sup>23</sup> and cryoprotection (<http://mousepheno.ucsd.edu/histo.shtml>) might be helpful.

12| Place enough 3.7% (vol/vol) formaldehyde on ice for more than 20 min. The solution's volume should be more than fivefold that of the tissue to be fixed.

▲ **CRITICAL STEP** The fixative solution (formaldehyde, 3.7% (vol/vol)) should be chilled, and collecting tubes and instruments should be prepared before euthanizing the mouse and dissecting the organ.

13| To repeat the experiment detailed herein, collect the most proximal section of the mouse intestine (duodenum) into a 15-ml conical Falcon tube containing 6–7 ml of fixative solution.

14| Dissect the tissue, and immediately place it into the chilled 3.7% (vol/vol) formaldehyde. Larger organs may be cut to smaller ( $3 \times 3 \times 3\text{ mm}$ ) pieces to facilitate quick penetration of the fixative solution into the depth of the tissue. Hollow organs, such as stomach, intestine or heart, should be flushed with cold fixative solution or cut open before they are submerged in the fixative solution in the tube. In this experiment, we cut the duodenum into 3- to 4-cm pieces, flushed them

## PROTOCOL

with cold 3.7% (vol/vol) formaldehyde and then transferred the pieces into a 15-ml conical tube containing 6–7 ml of cold 3.7% (vol/vol) formaldehyde.

▲ **CRITICAL STEP** Keep the dissected tissue on ice at all times. Perform all necessary tissue manipulations (such as flushing with fixative or cutting) in a sterile Petri dish with 2–3 ml of cold PBS in it, placed on ice.

**15|** To achieve tissue fixation, incubate the tubes for ~3 h at 4 °C with gentle agitation. If you are working with a thick tissue piece, consider longer incubation times, as the fixative penetration speed is ~1 mm h<sup>-1</sup>. In the current experiment, fix the mouse duodenum for 3 h.

**16|** (Optional) To improve the structural integrity of the sample, transfer the tissue into the tube filled with the cryoprotection solution (volume requirements are the same as for fixation). Incubate the mixture overnight at 4 °C with gentle agitation. We strongly encourage experimenters to perform this step.

### Preparation of frozen blocks ● **TIMING** 30–40 min

**17|** Label plastic molds for frozen block preparation with a permanent marker. If necessary, make marks on the mold to indicate the orientation of the tissue. Place the molds on ice, fill them with the Jung tissue freezing medium and let them chill for >5 min. Prechill 10 ml of PBS in a Petri dish placed on ice. Place another empty, open Petri dish on ice for manipulating the tissue. Prepare pieces of dry ice (flat slabs are the most convenient) in an appropriate container. We use styrofoam boxes with lids.

**18|** Remove the tissue from the fixative or cryoprotection solution, quickly rinse it by dipping it in the dish containing cold PBS, and then place it in the empty dish on ice. Cut the tissue to smaller pieces if necessary: for instance, we cut the intestine to pieces 3–5 mm long. The size of the embedded tissue pieces may vary depending on the tissue type and the researcher's preferences.

▲ **CRITICAL STEP** Keep the tissue cold at all times.

**19|** Place the tissue into the chilled molds filled with freezing medium. If necessary, fill the tissue cavities with medium by using a pipette. Orient the tissue as required for the most efficient sectioning. We position the intestine pieces to optimize obtaining cross-sections of the organ. When you pipette the freezing medium, avoid making air bubbles. If some bubbles are accidentally introduced, remove them by sucking them with a pipette into a 200-μl tip.

**20|** Place the molds with the tissue in Jung tissue freezing medium on dry ice. Allow it to solidify. If necessary, cover still exposed parts of the tissue with more medium. Allow it to stand on dry ice until the block is fully white and solid. Wrap the mold with the block in aluminum foil and label it.

■ **PAUSE POINT** The mold can be stored at –80 °C for years. Please note that after freezing, the block should never be allowed to thaw.

### Cryosectioning, fixation and permeabilization of the sections ● **TIMING** 5 h 30 min to overnight

**21|** Prepare the cover glasses and sharp-tip forceps (Equipment Setup). We use 22 × 22 (no. 1) coverslips and place them into six-well culture plates. Prepare dry ice (flat slabs are most convenient) in an appropriate container. We use styrofoam boxes with lids.

**22|** Set up the cryostat (Equipment setup) and clean the stage with 70% (vol/vol) ethanol. Use a new knife for sectioning. Place the tissue blocks inside the chamber, mount them and allow them to equilibrate to the temperature (15–30 min in case of small blocks, up to 1 h, if the blocks are large).

▲ **CRITICAL STEP** Do not let the blocks thaw or even soften.

**23|** Cut 5- to 10-μm sections and mount them onto the coated coverslips. Use sharp-ended forceps to pick up the coverslips. Let the sections air-dry for 3–5 min.

▲ **CRITICAL STEP** Drying time should be sufficient to allow the water present in the freezing medium to evaporate (insufficient drying will cause section detachment), but drying time should not exceed 5 min, as doing so may lead to RNA degradation. However, in a humid environment, it may be necessary to increase drying time to up to 8 min.

**24|** Place dried sections (on coverslips) into the wells of six-well plates on dry ice. It is important to keep the lids of the plate and the ice box closed, so as to prevent contact with warm room air and formation of water condensate on the coverslips. This condensation may cause section detachment. Keep sections on dry ice during sectioning procedure until

fixation (Step 25). Although sections can be kept at  $-80^{\circ}\text{C}$  or on dry ice for weeks, temperature in a freezer can accidentally rise (as a result of opening the door, for instance) and the sections may temporarily thaw, which will affect RNA integrity. We therefore do not recommend pausing the protocol at this point.

**25|** Remove the plates with sections on coverslips from the dry ice. Fix the sections by adding 2 ml of room-temperature 4% (wt/vol) paraformaldehyde into each well. At this stage, add 4% (wt/vol) paraformaldehyde dropwise, not directly onto each section, so as not to dislodge it from the glass. Incubate the sections for 15–20 min at room temperature.

**! CAUTION** Paraformaldehyde is highly toxic and carcinogenic; work should be carried out in a fume hood while you are wearing a lab coat and protective gloves.

#### ? TROUBLESHOOTING

**26|** Rinse the sections twice with room-temperature PBS, add 3 ml of 70% (vol/vol) ethanol to each well and incubate the mixture at  $4^{\circ}\text{C}$  for a minimum of 1 h (seal the plates with Parafilm to prevent ethanol evaporation). Proceed directly to Step 30, unless the resulting FISH signal is suboptimal, which may become evident at Steps 37 and 38.

**▲ CRITICAL STEP** Add liquids to the sections slowly, so as not to dislodge them from the glass.

**■ PAUSE POINT** Sections can be stored in 70% (vol/vol) ethanol at  $4^{\circ}\text{C}$  for several months.

#### Proteinase K digestion ● TIMING 20 min (optional)

**▲ CRITICAL** Steps 27–29 are optional, and proteinase K digestion should only be implemented if an insufficiently strong FISH signal is detected at Steps 37 and 38.

**27|** Rehydrate the sections by placing them into  $2\times$  SSC for 3–5 min.

**28|** Replace  $2\times$  SSC with proteinase K working solution and incubate the resulting mixture at room temperature for 5–10 min.

**29|** Wash the sections twice with  $2\times$  SSC.

#### Hybridization ● TIMING overnight

**30|** Pipette 2 ml of freshly made wash buffer into each well of a six-well plate, one well per coverslip, to rehydrate. Rehydrate the sections by picking them up with sharp-tip forceps and placing them, section side up, into wash buffer for 5 min.

**! CAUTION** Formamide is toxic and teratogenic. Handle it in a fume hood while you are wearing a lab coat and protective gloves.

**31|** Thaw the required quantity of hybridization buffer (100  $\mu\text{l}$  per  $22\times 22\text{-mm}$  coverslip) and set up the hybridization reactions according to option A, if you are counterstaining cell membranes with an antibody, or according to option B, if you are using Alexa Fluor 488 Phalloidin for that purpose instead (see also relevant discussion in the Experimental design section).

##### (A) Hybridization in the presence of an antibody for cell counterstaining

- (i) In a 1.5-ml microcentrifuge tube, combine the hybridization buffer, probes (from 1:100 intermediate dilutions, to the final dilution of 1:1,000–1:5,000) and the antibody solution. For instance, the mouse FITC-anti-E-cadherin antibody (1:100 dilution) may be used for counterstaining cell membranes in the intestine. In the case of the specific experiment described herein, prepare 1:100 intermediate dilutions in TE buffer of the *Lgr5*-TMR library, *Ki67*-Alexa Fluor 594 library and *Wipi1*-Cy5 library. Next, add 2  $\mu\text{l}$  of the intermediate dilution of the *Lgr5*-TMR intermediate dilution, 5  $\mu\text{l}$  of each intermediate dilution of libraries *Ki67*-Alexa Fluor 594 and *Wipi1*-Cy5 and 1  $\mu\text{l}$  of the mouse FITC-anti-E-cadherin antibody (undiluted) to 100  $\mu\text{l}$  of hybridization buffer. Mix and spin the mixture briefly.

**! CAUTION** Formamide is toxic and teratogenic. Handle it in a fume hood while wearing a lab coat and protective gloves.

##### (B) Hybridization with cell counterstaining without an antibody

- (i) Proceed as detailed in Step 31A. However, prepare the hybridization mix without the antibody.

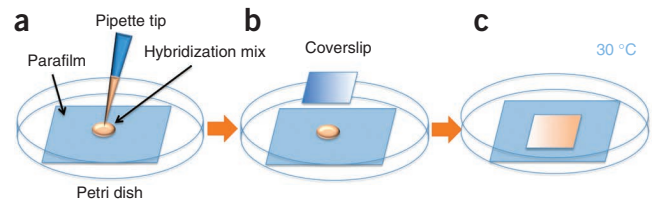
**! CAUTION** Formamide is toxic and teratogenic. Handle it in a fume hood while wearing a lab coat and protective gloves.

**32|** Spread a piece of Parafilm on the bottom of a Petri dish. Place 100- $\mu\text{l}$  drops of the hybridization mix onto a piece of Parafilm (**Fig. 4a**). Invert the coverslips, tissue side down, onto the drops (**Fig. 4b,c**). Avoid producing air bubbles in the process. Close the lid of the Petri dish. It is not necessary to humidify the inside of the dish. Perform hybridization overnight at  $30^{\circ}\text{C}$ .



## PROTOCOL

**Figure 4 |** Hybridization setup. (a) Spread Parafilm on the bottom of a Petri dish. Pipette 100  $\mu$ l of hybridization solution onto the Parafilm. (b) Place the coverslip with tissue section (tissue side down) onto the hybridization solution drop. (c) Cover the dish with lid and hybridize it overnight at 30 °C.



### Washing and mounting ● TIMING 1 h 40 min

**33|** Add drops of wash buffer under the coverslips and then lift them off the hybridization solution carefully. Place the coverslips back into the six-well plate, rinse them with 2 ml of wash buffer per well, add fresh wash buffer and wash them by incubating at 30 °C for 30 min. Please note that agitating the plate is not necessary.

**! CAUTION** Formamide is toxic and teratogenic. Handle it in a fume hood while wearing a lab coat and protective gloves.

**34|** Replace the first wash buffer with fresh wash buffer containing DAPI and incubate it for 30 min at 30 °C.

**! CAUTION** Formamide is toxic and teratogenic. Handle it in a fume hood while wearing a lab coat and protective gloves.

**35|** Equilibrate the hybridization mixtures according to option A, if you opted to stain cell borders with mouse FITC–anti-E-cadherin antibody, or according to option B, if you opted to use Alexa Fluor 488 Phalloidin.

#### (A) Equilibration in the presence of an antibody for cell counterstaining

(i) Place each coverslip in 2 ml of GLOX buffer (with no enzymes) and allow it to equilibrate for >5 min.

■ **PAUSE POINT** The hybridized tissue sections can be kept in GLOX buffer, without enzymes, at 4 °C for up to 24 h without any adverse effects on smFISH signal quality.

#### (B) Equilibration in the presence of Alexa Fluor 488 phalloidin

(i) Place each coverslip in 2 ml of GLOX with Alexa Fluor 488 phalloidin. Incubate the coverslips for 15 min, and then transfer each coverslip to 2 ml of fresh GLOX buffer with no enzymes. Incubate the coverslips for 5 min.

#### ? TROUBLESHOOTING

■ **PAUSE POINT** The hybridized tissue sections can be kept in GLOX buffer, without enzymes, at 4 °C for up to 24 h without any adverse effects on smFISH signal quality.

**36|** Place 15  $\mu$ l of imaging buffer onto a microscope slide. Lay each coverslip, hybridized tissue section down, onto the drop. Avoid producing air bubbles in the process. Suck any excess fluid off with a piece of filter paper, being careful not to move the coverslip. Seal the edges with rubber cement, and let it air-dry for 10–20 min.

▲ **CRITICAL STEP** Imaging buffer must be freshly prepared and used immediately or kept on ice for a maximum of 8 h.

### Microscope setup and data collection ● TIMING 4–6 h

**37|** Mount the slide onto the microscope stage and find the right focal plane for the z-middle of the tissue section under DAPI illumination. Define xy positions to image the regions of interest (intestinal crypts, in our case). Set up acquisition of z-stacks, covering a thickness of the tissue in an optimal way. In the case of intestines, acquire 2–5- $\mu$ m-thick stacks.

▲ **CRITICAL STEP** The distance between individual optical planes should be 0.2–0.4  $\mu$ m.

#### ? TROUBLESHOOTING

**38|** Acquire z-stacks at each xy position in each wavelength channel, starting with Cy5, then Alexa Fluor 594, then TMR, then FITC and then DAPI. Typical illumination times are 0.5–3 s for Cy5, Alexa Fluor 594 and TMR, 50–200 ms for FITC and 20–30 ms for DAPI. However, illumination times should be adjusted for each slide to obtain optimal results. Please avoid excessive exposure times, which will be defined by an observed saturation of pixel intensities. For the experiment described here, implement the following exposure times: 1 s for Cy5, 3 s for Alexa Fluor 594, 500 ms for TMR, 200 ms for FITC and 25 ms for DAPI. The number and relative locations of xy positions at which z-stacks will be acquired are determined by tissue architecture, by the number of cells expressing mRNA of interest and by the numbers of mRNA molecules of interest. The number of acquired xy positions should be optimal to enable statistical analysis, and it may vary in different tissues and for different mRNA species. The xy positions at which z-stacks will be acquired can be chosen either manually or automatically (for example, scan and image certain areas of the sample using the imaging software's scanning tool; we use Metamorph, in this case). In the case of intestinal crypts, record images of 30–40 crypts in stacks of 2–3  $\mu$ m thickness, with 0.3  $\mu$ m between individual planes.

▲ **CRITICAL STEP** In any smFISH experiment, make sure to always acquire light emission data from less-stable fluorophores (or from probes, experimentally shown to give weaker signal, fused to any fluorophore) first. With the channels used in this protocol, we recommend the following order: Cy5, Alexa Fluor 594, TMR, FITC, DAPI, bright field. This order can be modified empirically, by evaluating the performance of each fluorophore-fused probe library in a single-color experiment. Please note

that here we acquire FITC data after Cy5, Alexa Fluor 594 and TMR, even though FITC is less stable than the other fluorophores. The reason for doing so is that FITC is only used for cell boundary staining, and not for the more sensitive smFISH data acquisition. This order of acquisition is chosen to minimize the effect of bleaching, which results from repeated illumination during stack acquisition in other channels.

## ? TROUBLESHOOTING

**39|** Record data in TIFF format. For automated FISH signal detection (single-molecule RNA counting), we use ImageM, our custom-written MATLAB-based software (see **Box 1** for directions). Automatic transcript detection uses algorithms described previously<sup>2,3</sup>. The spot stack images are first filtered with a 3D Laplacian of Gaussian filter of 15 pixels in size and a s.d. of 1.5 pixels. The number of connected components in binary-thresholded images is then recorded for a uniform range of intensity thresholds, and the threshold for which the number of components was least sensitive to threshold selection is used for spot detection. At this stage, dots appearing in multiple fluorescence channels are automatically detected and can be removed by the software (**Box 1**). These dots are often nonspecific aggregates of probes or autofluorescent organelles. We advise removing such dots. Please note that images from different fluorescence channels may have a slight shift, depending on the imaging setup. The use of zero-shift filter cubes can alleviate this problem, however, and if a shift exists it can be corrected by software. Beads fluorescing in all channels can be used to detect and characterize the shifts between channels. Additional filtering of spots, which are not single transcripts, can include removal of spots exceeding a certain size threshold, the size expected from a diffraction-limited object, which is associated with a single mRNA molecule.

## ? TROUBLESHOOTING

**40|** Automatic cell segmentation can be performed by relying on DAPI channels in some cases (e.g., using CellProfiler<sup>24</sup> or similar software). In tissues such as intestinal crypts, the nonisotropic cell morphology limits the precision of such approach and requires manual cell border segmentation. We advise readers to use our ImageM software (**Box 1**) to perform manual cell segmentation, on the basis of both DAPI and E-cadherin antibody staining. In intestinal crypt tissue, we segment only one cell for each vertical position along the crypt-villus axis, as was done in the example experiment (**Fig. 1f**).

**41|** Assign the detected mRNA-molecule spots to cells to generate single-cell gene expression signatures. Use either the discrete integer number of transcripts per cell or, alternatively, the transcript concentration, which is defined as the number of transcripts divided by cell volume and estimated as the segmented cell area times the number of analyzed optical z-sections (planes). Please note that transcript concentration estimation can be a more robust feature when you pool results from several experiments in which the numbers of analyzed planes are different.

**42|** Perform gene expression analysis of choice. An example of such analysis in the intestinal crypt is creation of spatial expression profiles along the axis of the crypt (bottom to top). The result of the example experiment (a typical result of such profiling of multiple crypts) is represented in **Figure 1g,h**.

## ? TROUBLESHOOTING

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

**TABLE 1 |** Troubleshooting table.

Step	Problem	Possible reason	Solution
25–35	Tissue sections detach from the glass	Insufficient cleaning and/or coating of the coverslips	Increase the length of coverslip sonication in detergent and ethanol to 20 min or add a second sonication step in ethanol. Make sure the coated coverslips are used immediately, or that they are stored in the desiccator only
		Insufficient drying at Step 23	Increase drying time by 1–2 min. Try not to exceed 8 min
37	Tissue integrity is poorly preserved, with frequent rips and holes throughout the samples	Tissue is cryodamaged	Cryoprotection step is necessary (Step 16)

(continued)

**TABLE 1** | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
	Tissue is twisted and/or ripped	Sample was damaged during mounting (Step 36)	Do not let coverslip move during mounting
38, 39	Insufficient signal intensity (low signal-to-background ratio)	Nonspecific probe binding owing to a suboptimal formamide concentration, leading to a high background fluorescence	Increase formamide concentration up to 25% (vol/vol) in wash and hybridization buffers (the required formamide concentration can be probe dependent)
		Insufficient number of binding probes due to the association of target mRNA with proteins	Subject tissue sections to proteinase K digestion (Steps 27–29). Increase proteinase K concentration, the duration of digestion and the incubation temperature, if needed, being careful not to affect tissue integrity
		Insufficient number of binding probes due to a small oligonucleotide library size	If possible, increase the size of the library, such as by adding UTRs to the target sequence
		High autofluorescence	Use red dyes, such as Cy5 instead of AlexaFluor594 and TMR. Autofluorescence is generally weaker at longer wavelengths, improving signal-to-noise
	Photobleaching (signal quickly fades during acquisition)	Antiphotobleaching medium is not working properly	Prepare fresh GLOX buffer. Make sure glucose oxidase and catalase are fresh, properly reconstituted and stored. Activity of these enzymes is crucial to buffer performance Use a lower intensity of excitation light, in particular when using laser illumination in (spinning-disk) confocal microscopy
	Intensity of fluorescent dots decays toward the edges of the image, rendering accurate quantification difficult	Uneven illumination of the sample (vignetting)	Optimize alignment of the illumination, apply digital shading correction, or exclude peripheral areas from analysis

## TIMING

Steps 1–5, coupling FISH probe libraries to fluorophores: 2–4 h  
Steps 6–10, probe precipitation, washing and purification: 1–2 d  
Step 11, probe reconstitution and storage: 5–10 min  
Steps 12–16, tissue dissection, fixing and cryoprotection: 4 h to overnight  
Steps 17–20, preparation of frozen blocks: 30–40 min  
Steps 21–26, cryosectioning, fixation and permeabilization of the sections: 5 h 30 min to overnight  
Steps 27–29, proteinase K digestion (optional): 20 min  
Steps 30–32, hybridization: overnight  
Steps 33–36, washing and mounting: 1 h 40 min  
Steps 37–42, microscope setup and data collection: 4–6 h

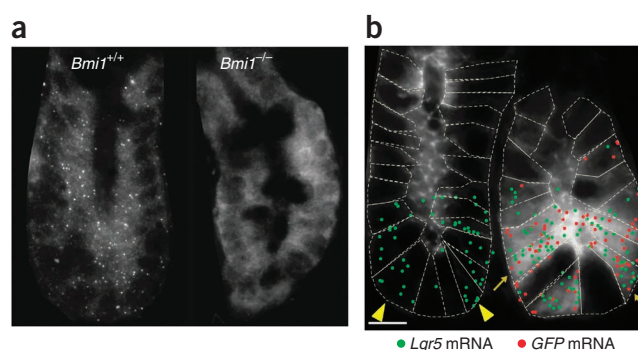
## ANTICIPATED RESULTS

This protocol enables users to implement smFISH in a variety of tissues. It enables assessing the absolute numbers of mRNA molecules in single cells in the context of a tissue. The mRNA expression is automatically quantified, semiautomatically assigned to single cells in the tissue and analyzed, resulting in high-resolution gene expression maps of the tissue.

Results of a typical three-color smFISH experiment (with mouse intestine as a target tissue) are shown in **Figure 1**. Diffraction-limited spots are clearly observed by microscopy. First, the smFISH spots are recognized and counted by our



**Figure 5** | Positive and negative controls for smFISH. (a) smFISH detection of *Bmi1* expression in a wild-type mouse (left panel, serves as a positive control for the method) and a *Bmi1*-knockout mouse (right panel, serves as a negative control). (b) smFISH detection of *Lgr5* mRNA (green dots) and *GFP* mRNA (red dots). Arrows indicate cells expressing both *Lgr5* and *GFP* transcripts and showing GFP fluorescence (positive control for GFP smFISH). Arrowheads denote GFP fluorescence-negative cells, devoid of *GFP* transcripts, but containing *Lgr5* mRNAs (negative control for GFP smFISH). Figure modified with permission from Itzkovitz *et al.*<sup>3</sup>



MATLAB software (ImageM). Only the signal coming from the binding of the probe library to a single mRNA molecule (specific signal) is recognized and taken into account by the software. A specific signal (i.e., coming from the binding of the probe library to a single mRNA molecule) must meet the following criteria: (i) there must be sufficient signal-to-background ratio (of ~2 in a typical experiment); (ii) the size of the signal spot must correspond to the size of a diffraction-limited spot (200–300 nm, which in our setup corresponds to ~4–6 pixels); and (iii) the signal spot must appear only in the appropriate fluorescence channel. Fluorescent spots that appear in multiple channels are filtered out by ImageM. For example, in the experiment used as template in this protocol, large blots of fluorescence, resulting mostly from nonspecific binding of the probes, are present (Fig. 1a–c, arrows) alongside specific signals (Fig. 1a–c, arrowheads). These spots are clearly distinguishable from the specific signal by their size (larger than diffraction-limited spots), and by the fact that, unlike specific signal, they usually appear in all three fluorescent channels. Therefore, these nonspecific fluorescent spots are not present in the software-generated representation of the results (Fig. 1f).

The use of positive and negative controls will greatly improve reliability of the protocol. Fluorescence spots, resulting from nonspecific binding of the probes, will be present in both controls. Different cell types, one expressing and another not expressing the mRNA of interest, can serve as positive and negative controls, respectively. For example, intestinal crypt base columnar cells express *Lgr5* mRNA, whereas enterocyte or Goblet cells do not express *Lgr5* mRNA (Fig. 1a). Another example includes *Ki67*, a proliferation marker expressed only in dividing cells within the crypt, and not in the differentiated cells in the villi (Fig. 1b).

Tissues known to express or not to express an mRNA of interest can be used as a positive and negative control, respectively. We present here an example of such controls for the smFISH probe library against *Bmi1* mRNA, where *Bmi1* is a gene expressed in intestinal crypts. A wild-type crypt is used as a positive control, and a crypt derived from a *Bmi1*-knockout mouse is used as a negative control (Fig. 5a)<sup>3</sup>. smFISH against an exogenous transcript (such as GFP), expressed in mammalian cells or tissue, can serve as an excellent positive or negative control for the protocol. GFP is especially convenient in this context, as its presence is easily detected by green fluorescence. GFP-positive fluorescent cells also contain GFP mRNAs (Fig. 5b, arrows), whereas cells devoid of fluorescence are GFP transcript negative (Fig. 5b, arrowheads)<sup>3</sup>.

Next, manual definition of cell borders (cell segmentation) is performed (Figs. 1f and 2). Although the procedure is made user-friendly by our GUI (ImageM), it is the most time-consuming step in the process of the analysis. Finally, each of the segmented cells is assigned a number and detected spots are assigned to the cells. On the basis of this assignment, expression of the three genes can be presented in the context of the crypt (Fig. 1g,h).

Note: Supplementary information is available in the online version of the paper.

**AUTHOR CONTRIBUTIONS** A.v.O. supervised the project. A.L., S.I. and J.P.J. developed the protocol. X.W. and Z.P.F. contributed to software development. A.L., S.I., J.P.J. and A.v.O. wrote the paper.

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