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Title: Probing mRNA Kinetics in Space and Time in *Escherichia coli* Using Two-Color Single-Molecule Fluorescence In Situ Hybridization

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Author Questionnaire

1. Microscopy: Does your protocol demonstrate the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **56**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Sangjin Kim**: smFISH is known for measuring the absolute number and subcellular location of mRNAs. Our two-color smFISH protocol has the added capability of measuring the kinetics of transcription and mRNA degradation **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Kavya Vaidya**: Using this method, many samples can be processed simultaneously without extra time or effort. For example, four time-course experiments, yielding 48 samples, can be processed for imaging in 8 hours **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Kavya Vaidya**: Our protocol is broadly applicable for many genes and bacterial species **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

Protocol

2. Coverslip and Glass Slide Preparation

2.1. To prepare coverslips and glass slides for the experiment, use forceps to place coverslips and slides in a Coplin jar containing 100% ethanol **[1]** and place the jar in a water-bath ultrasonicator for 15-20 minutes **[2]**.

2.1.1. WIDE: Talent placing slides/coverslips into jar, with ethanol container visible in frame
2.1.2. Jar being sonicated

2.2. At the end of the sonication, wash the slides and coverslips 3-4 times with ultrapure water **[1]** before re-filling the jar with 70% ethanol and repeating the sonication **[2]**.

2.2.1. Talent washing jar with ultrapure water
2.2.2. Talent placing jar in sonicator, with ethanol container visible in frame

2.3. After the second sonication, dry the coverslips and slides with nitrogen gas **[1 and 2-added]**. Place the coverslips into an empty 1000-microliter pipette tip box **[4]** and place the slides into a clean slide box **[3]**.

2.3.1. Talent blow drying coverslip
2.3.2. ADDED SHOT: ZOOM version of 2.3.1.
2.3.3. Talent placing slides into slide box NOTE: Show 4 before 3, VO changed to match.
2.3.4. Talent placing coverslips into chamber

2.4. Then, following the holes of the pipette tip box, use a hydrophobic marker to draw circles onto the coverslips **[1]** and apply a 20 microliter drop of 0.1% poly-L-lysine to each of these "wells" **[2]**.

2.4.1. Talent drawing circles on the coverslip Author NOTE: Use the zoomed in version. *Videographer: Important step*
2.4.2. Talent applying poly-L-lysine to a well, with poly-L-lysine container visible in frame *Videographer: Important step*

3. Time-Course Experiment and Sample Fixation

3.1. To set up a time-course experiment, add 750 microliters from a 20-milliliter exponentially growing *E. coli* culture **[1]** to a 1.5-milliliter culture tube marked "time zero" **[2-TXT]** and immediately invert the tube **[3]**.

3.1.1. WIDE: Talent aspiring bacteria from flask

3.1.2. Talent adding bacteria to a tube, marked "time zero" **TEXT: See text for all medium and solution details**

3.1.3. Tube being inverted

3.2. Induce *lacZ* (lack-z) expression with 0.2-1-millimolar IPTG (I-P-T-G) **[1-TXT]** and start a timer **[2]**.

3.2.1. Talent adding IPTG to flask, with IPTG container visible in frame TEXT: **IPTG: isopropyl beta-D-1-thiogalactopyranoside**

3.2.2. Timer being started, with "0:00" in the timer visible in frame

3.3. After checking the timer, collect the cell culture at each consecutive experimental time point as just demonstrated **[1]**.

3.3.1. Talent aspiring bacteria from the flask, with tube labeled t= 1 visible in frame
Videographer: Important step

3.4. At the appropriate time point during the experiment, add 5-millimolar ONPF (O-N-P-F) to the flask to repress *lacZ* expression **[1-TXT]** and continue to sample the cultures to track the mRNA degradation **[2]**.

3.4.1. Talent adding ONPF to flask, with OPNF container visible in frame **TEXT: ONPF: orthonitropheynl-beta-D-fucopyranoside**

3.4.2. Talent adding bacteria to tube

3.5. At the end of the time-course sample acquisition, incubate the samples at room temperature for 15 minutes **[1]** followed by incubation on ice for 30 minutes **[2]**.

3.5.1. Talent setting timer, with sample tubes visible in frame

3.5.2. Talent placing tube(s) on ice

3.6. At the end of the incubation, centrifuge the samples to remove the fixative **[1-TXT]** and use a pipette to remove the supernatant **[2]**.

3.6.1. Talent placing tube(s) into centrifuge **TEXT: 4 min, 4500 x g, RT**

3.6.2. Shot of pellet(s) if visible, then supernatant being removed Author NOTE: Pellet was photographed using a macro-lens. Perhaps, this should be two scenes, one is the macro-lens shot and the other is removing supernatant.

3.7. Resuspend the bacteria in 1 milliliter of DEPC (D-E-P-C)-PBS **[1-TXT]** and wash the cells two more times in 1 milliliter of fresh DEPC-PBS per wash **[2]**.

3.7.1. Talent adding DEPC-PBS to tube, with DEPC-PBS container visible in frame and mix by pipette. **TEXT: DEPC: diethylpyrocarbonate**

3.7.2. ~~Talent adding tube(s) to centrifuge~~ NOTE: reuse 3.6.1.

3.8. After the last wash, resuspend the cells in 30 microliters of fresh DEPC-PBS **[1]**.

3.8.1. Shot of tube(s), then cells being pipetted up and down, with DEPC-PBS container visible in frame

4. Cell Membrane Permeabilization

4.1. For permeabilization of the cells, first add the sample from each time point to individual hydrophobic wells on one of the ethanol-sterilized, glass coverslips **[1-TXT]** and allow the cells to adhere to the coverslip with a 10-30-minute incubation at room temperature **[2]**.

4.1.1. WIDE: Talent adding sample(s) to well(s), with wells and sample tubes visible in frame *Videographer: Important step* **TEXT: See text for full coverslip preparation details**

4.1.2. Talent setting timer, with chamber visible in frame *Videographer: Important step*

4.2. At the end of the incubation, add 15 microliters of 70% ethanol to each well **[1]**. After 4 minutes, aspirate the ethanol from each well so that the wells are completely dry **[2]**.

4.2.1. Talent adding ethanol to well(s), with wells, timer, and ethanol container visible in frame *Videographer: Difficult step*

4.2.2. Ethanol being aspirated **TEXT: All ethanol should be removed** NOTE: Please use take 3 *Videographer: Difficult step*

5. Probe Hybridization

5.1. After washing, add 30 microliters of pre-hybridization solution to each well of permeabilized cells **[1]** and incubate the cells for 30 minutes in a 37-degree Celsius oven **[2]**.

5.1.1. WIDE: Talent adding solution to well(s), with solution container visible in frame

5.1.2. Talent storing the chamber in oven

5.2. Replace the pre-hybridization solution with approximately 30 microliters of probe hybridization solution per well **[1]** and cover the chamber with aluminum foil for a 2-hour incubation in the 37-degree Celsius oven **[2]**.

5.2.1. Hybridization solution being added to well(s), with solution container visible in frame *Videographer: Difficult step*

5.2.2. Talent putting the foil-covered chamber into oven

6. Post-Hybridization Wash and Imaging Preparation

6.1. At the end of the incubation, use a multichannel pipette to rinse the wells 3-5 times with 30 microliters of wash solution per well per wash **[1-TXT]**.

6.1.1. WIDE: Talent washing well(s), with solution container visible in frame **TEXT: Aspirate by vacuum after each wash** *Videographer: Important step*; *Videographer/Video Editor: shot will be used again*

6.2. After the last wash, incubate the cells for 15-30-minute at 37 degrees Celsius **[1]** before washing the wells five times with 30 microliters of fresh DEPC-PBS per wash **[2]**.

6.2.1. Talent placing box into incubator *Videographer: Important step*

6.2.2. DEPC-PBS being added to well(s), with DECP-PBS container visible in frame *Videographer: Important step*

6.3. After the last wash, aspirate all of the liquid from the coverslip **[1]** and add 4 microliters of fresh DEPC-PBS to each well **[2]**.

6.3.1. Liquid being aspirated

6.3.2. DEPC-PBS being added to well(s)

6.4. Use forceps to carefully place the coverslip onto an ethanol-sterilized glass slide sample-side down **[1]** and use silicone dental gum to seal the edges of the coverslip **[2-TXT]**.

6.4.1. Coverslip being inverted/placed onto slide *Videographer: Important step*

6.4.2. Edges being sealed *Videographer: Important step* **TEXT: Optional: Store sealed coverslip at 4 °C**

Author NOTE: This step was not done very well during the shoot. The sealant is just too messy. After the videographer left, we filmed this step by ourselves. The video file is uploaded as " 61520_Step_6.4.2.MOV". Please use this if it looks nice.

If you have to use the video taken by the videographer, please add a text as

TEXT: Remove overflown sealants using a razor blade

And show (61520_Step_6.4.2a = before the removal and 61520_Step_6.4.2b = after the removal)

7. Imaging

7.1. To locate an area of interest, select the live mode of phase contrast imaging **[1]** and maneuver the stage joystick to change the field of view within a well **[2]**.

7.1.1. WIDE: Talent selecting live mode, with monitor visible in frame

7.1.2. SCREEN: screenshot_1: 00:13-00:30 *Video Editor: please speed up*

7.2. Locate an area in which the cell density is optimal **[1]** and adjust the z-focus such that the phase-contrast cell images are in focus **[2]**.

7.2.1. SCREEN: screenshot_2: 00:08-00:24 *Video Editor: please speed up*

7.2.2. SCREEN: screenshot_2: 00:24-00:32

7.3. Then acquire a Cy5 (sigh-five) image with a 4-second exposure **[1]**, a Cy3 image with a 2-second exposure **[2]**, and a phase contrast image with a 0.2-second exposure for approximately 10 different areas per well **[3]**.

7.3.1. SCREEN: screenshot_3: 00:02-00:13 *Video Editor: can speed up*

7.3.2. SCREEN: screenshot_3: 00:14-00:20

7.3.3. SCREEN: screenshot_3: 00:21-00:26

7.4. Alternatively, an ND acquisition, in which Cy5, Cy3, and phase contrast images are acquired in series, can be run **[1]**.

7.4.1. SCREEN: screenshot_4: 00:03-00:15

8. Image Analysis

8.1. When all of the wells have been imaged, open an appropriate cell segmentation tool **[1]** and unclick **Stack [2]**.

8.1.1. WIDE: Talent opening tool, with monitor visible in frame

8.1.2. SCREEN: screenshot_5: 00:00-00:03

8.2. Load the phase contrast images of interest **[1]** and select **Independent frames** and click **Compute Phase Profile as 0 [2]**.

8.2.1. SCREEN: screenshot_5: 00:03-00:10

8.2.2. SCREEN: screenshot_6: 00:00-00:04

8.3. To begin the segmentation process, during which the cells will be identified and their contours will be calculated, load the parameters provided in the GitHub folder and click **All frames [1]**.

8.3.1. SCREEN: screenshot_6: 00:05-00:16

8.4. At the end of the processing, select **Contour** to visualize the mesh **[1]**.

8.4.1. SCREEN: screenshot_6: 00:26-00:28

8.5. Next, under the **spotDetection** tab, uncheck **Stack**, check **Meshes** and click **Rub** to begin the spot identification and quantification based on the 2D Gaussian fitting **[1]**.

8.5.1. SCREEN: screenshot_7: 00:00-00:13

8.6. Select the folder containing the fluorescent images and the mesh file that was just calculated from the cell detection procedure and indicate the file name to which the spotDetection result will be saved **[1]**.

8.6.1. SCREEN: screenshot_7: 00:14-00:34 *Video Editor: please speed up*

8.7. Then use the image analysis workflow provided on GitHub to analyze the fluorescence images **[1]**.

8.7.1. SCREEN: screenshot_8: 00:07-00:20 **TEXT: https://github.com/sjkimlab/Code_Publication/tree/master/JoVE_2020**

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.4., 3.3., 4.1., 5.1., 5.2., 5.4.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
4.2., 5.2.

Results

9. Results: Representative mRNA Kinetic Probing Analysis

9.1. In this full field of view, approximately 500 *E. coli* cells at a good density for cell segmentation can be observed **[1]**.

9.1.1. LAB MEDIA: Figure 3A

9.2. The morphology of the cells in the phase contrast images should remain comparable to that of live cells for segmentation purposes **[1]**.

9.2.1. LAB MEDIA: Figures 3B and 3C top rows

9.3. If the cells are over-permeabilized, their morphology will become unsuitable for segmentation **[1]**.

9.3.1. LAB MEDIA: Supplementary Figure 1

9.4. The distribution of *lacZ* mRNA spot intensities before induction **[1]** does not fit well with a normal or Poisson distribution due to the presence of spots with high intensities **[2]**.

9.4.1. LAB MEDIA: Figures 3D and 3E
9.4.2. LAB MEDIA: Figures 3D and 3E *Video Editor: please emphasize light green/grey histogram data lines in both graphs*

9.5. When the expression of *lacZ* is induced **[1]**, the signal of 5-prime *lacZ* mRNA increases before **[2]** the 3-prime *lacZ* mRNA signal increases **[3]**.

9.5.1. LAB MEDIA: Figure 4B
9.5.2. LAB MEDIA: Figure 4B *Video Editor: please emphasize red 5' data line*
9.5.3. LAB MEDIA: Figure 4B *Video Editor: please emphasize green 3' data line*

9.6. If the expression of *lacZ* is repressed, both the 5- and 3- *lacZ* mRNA signals decrease **[1]**.

9.6.1. LAB MEDIA: Figure 4B *Video Editor: please emphasize red and green data lines on right side of peaks*

9.7. To obtain the rate of transcription elongation, the rise of the 5- and 3-prime signals are fit with lines **[1]**, while the rate of mRNA degradation is obtained by fitting the decay region with an exponential function **[2]**.

9.7.1. LAB MEDIA: Figure 4B *Video Editor: please emphasize fit lines for both red and green data lines*

9.7.2. LAB MEDIA: Figure 4B *Video Editor: please emphasize decay fit lines for both red and green data lines*

9.8. Because single molecule FISH (fish) is a single-cell technique, cell-to-cell variabilities in transcription can also be analyzed **[1-TXT]**.

9.8.1. LAB MEDIA: Figure 4C **TEXT: FISH: fluorescence in situ hybridization**

9.9. In addition, analysis of the colocalization of 5 prime and 3 prime *lacZ* mRNA **[1]** allows the density of the RNA polymerases on the *lacZ* gene to be quantified **[2]**.

9.9.1. LAB MEDIA: Figure 5B *Video Editor: please add/emphasize purple arrow*

9.9.2. LAB MEDIA: Figure 5C *Video Editor: please emphasize grey dots*

Conclusion

10. Conclusion Interview Statements

10.1. **Kavya Vaidya**: As samples from different time points are handled simultaneously during this procedure, it is important to keep the samples separated while they are in the tubes and on the coverslip **[1]**.

10.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

10.2. **Sangjin Kim**: Using this single-molecule, single-cell microscopy technique, researchers can explore how transcription and degradation kinetics are related to the absolute numbers or subcellular localization of mRNAs **[1]**.

10.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*