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Title: Single Molecule Fluorescence Energy Transfer Study of Ribosome Protein Synthesis

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, done**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations?

Current Protocol Length

Number of Steps: 18
Number of Shots: 55

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Yuhong Wang:** Single molecule FRET can capture dynamics happening at nanometer distances, which is the right scale for ribosomal protein synthesis process.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *B-roll 4.3.1.*

1.2. **Yuhong Wang:** Ribosome works by coordinating multiple factors and components allosterically, which are intrinsically inhomogeneous. Single molecule method can track each ribosome without being limited by this inhomogeneous average effect.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *B-roll 4.7.2.*

OPTIONAL:

1.3. **Yuhong Wang:** This method will reveal how antibiotics inhibit ribosome function to develop new drugs toward drug-resistant bacterial infections.

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

Protocol

2. Preparation of the Ribosome Complexes

2.1. Begin by preparing the PRE by mixing the initiation-, EF-Tu_NoG-, and Phe- mix, at the ratio of 1 to 2 to 2 at 37 degrees Celsius for 2 minutes **[1-TXT].** After incubation, purify the PRE using 1.1 molar sucrose cushion ultracentrifugation overnight at 100,000 x *g* **[2]**. *Videographer: This step is important!*

2.1.1. WIDE: Establishing shot of the talent mixing the initiation mixes. **TEXT: PRE- pre-translocation complex**

2.1.2. Talent placing the PRE for centrifugation.

NOTE: Videographer strickthrough shot 2.1.2.

2.2. Prepare the POST by mixing the initiation-, EF-Tu_WG- (pronounce 'EF-Tu_WG' as it is spelled) , and Phe- mix, at the ratio of 1 to 2 to 2 at 37 degrees Celsius for 10 minutes **[1-TXT]**. After incubation, purify the POST using 1.1 molar sucrose cushion ultracentrifugation overnight at 100,000 x g **[2]**.

2.2.1. Talent mixing the initiation mixes. **TEXT: POST- post-translocation complex**

2.2.2. Talent placing the tubes for centrifugation. Videographer's NOTE for 2.2.2: Both samples at the same time.

3. Preparation of Sample Slides

3.1. Clean the microscope glass slides containing six pairs of holes that are 1 millimeter in diameter and the number 1.5 microscope glass coverslips **[1]**. Bake the cleaned slides and coverslips at 300 degrees Celsius for 3 hours **[2]**, then coat the coverslip with aminosilane **[3]**. *Videographer: This step is important!*

3.1.1. Talent cleaning the microscope glass slides and glass coverslips.

3.1.2. Talent baking the slides and the coverslips.

3.1.3. Talent coating the coverslip with aminosilane.

3.2. In a clean laminar hood, lay one coverslip flat on the surface **[1]**. Carefully drop 60 microliters of PEG solution on the top edge **[2]**. Then, lay another coverslip on top of it **[3]**, letting the capillary effect spread the solution between them, ensuring that no bubbles are formed. Repeat these steps for 2 more pairs of coverslips **[4]**.

3.2.1. Talent placing one coverslip flat on the surface.

3.2.2. Talent dropping PEG on the top edge.

3.2.3. Talent placing the other coverslip on top of the one lying flat.

3.2.4. A shot of the coverslips as the solution spread between them.

Videographer's NOTE: 3.2.1 to 3.2.4 were filmed in one shot and labeled as 3.2.1.

NOTE: Milliliter was changed with microliter in VO narration for step 3.2 by the authors.

3.3. Store these coverslips in an empty tip-box filled with water and incubate them for 3 hours in the dark **[1]**. After 3 hours, separate the coverslips **[2]**, rinse with water in three consecutive crucibles **[3]** and purge with a dry nitrogen stream **[4]**. *Videographer: This step is important!*

3.3.1. Talent placing the coverslips in the tip-box.
3.3.2. Talent separating the coverslips.
3.3.3. Talent rinsing the coverslips in the crucibles.
3.3.4. Talent purging with dry nitrogen steam.

Videographer's NOTE: 3.3.2 to 3.3.4 were filmed in one shot and labeled as 3.3.2.

3.4. Pull sharp pipette tips through the holes on the glass slides until they fit tightly **[1]**. Scrape the sharp ends off until they are completely flat with the glass surface **[2]**. Then, cut a double-faced tape with the channel pattern on it **[3-TXT]**. Stick the tape onto the glass slides on the flat side **[4]**.

3.4.1. ECU: Talent pulling the sharp pipette tips through the holes.
3.4.2. Talent scraping the sharp ends off.
3.4.3. Talent cutting the double-faced tape. **TEXT: 25 x 40 mm**
3.4.4. Talent sticking the tape onto the glass slide.

Videographer's NOTE: 3.4.2 to 3.4.4 were filmed in one shot and labeled as 3.4.2.

3.5. Stick the coated coverslip onto the double-faced tape **[1]** and press on it to make a tight seal of the sample chamber, making sure the coated side is facing inward **[2]**.

3.5.1. Talent sticking the coated coverslip onto the double-faced tape.

3.5.2. Talent pressing on it to make a tight seal of the sample chamber.
Videographer's NOTE: 3.5.1 and 3.5.2 were filmed in one shot and labeled as 3.5.1.

4. Single-Molecule FRET Imaging

4.1. Turn on the computer and the microscope switch **[1]**. Start the laser control interface **[2]** and turn on the laser **[3]**. Push the enable button on the laser control box to warm up the laser **[4]**.

4.1.1. Talent turning on the computer and microscope.
4.1.2. Talent starting the laser interface.
4.1.3. Talent turning on the laser.
4.1.4. Talent pushing the enable button on the control box.

Videographer's NOTE: 4.1.3 and 4.1.4 were filmed in one shot and labeled as 4.1.3.

4.2. Turn on the cameras **[1]**. Then, start the microscope-associated software program **[2]**. Click the upper shutter **Off** and click the lamp **On** **[3]**.

4.2.1. Talent turning on the cameras. Videographer's NOTE for 4.2.1: Use 4.1.1.
4.2.2. SCREEN: 62664.4.2.2&3.mp4. 00:04 – 00:06, 00:40 – 00:45
4.2.3. SCREEN: 62664.4.2.2&3.mp4. 00:47 – 00:52

4.3. Prepare the TAM_{10}_WT (pronounce 'Tam-ten with tween') buffer by adding 10 microliters of 5 percent tween20 into 1 milliliter of TAM_{10} buffer **[1]**. Prepare the deoxy solution by weighing 3 milligrams of glucose oxidase in a small microcentrifuge tube **[2]** and add 45 microliters of catalase to it **[3]**. *Videographer: This step is important!*

4.3.1. Talent adding tween20 to the TAM_{10} buffer.
4.3.2. Talent preparing the deoxy solution.
4.3.3. Talent adding catalase to the tube.

Videographer's NOTE: 4.3.1 to 4.3.3 were filmed in one shot and labeled as 4.3.1.

NOTE: Milliliter was changed with microliter in VO narration for step 4.3 by the authors.

4.4. Vortex the tube gently to dissolve the solid **[1]**. Spin at 20,000 x g in a microcentrifuge for 1 minute and take the supernatant **[2]**.

4.4.1. Talent vortexing the tube.
4.4.2. Talent spinning down the solution.

4.5. Prepare 30 percent glucose solution, 200 millimolar Trolox solution, and 0.5 milligrams per milliliter of streptavidin solution following instructions in the text manuscript **[1]**. Add one drop of imaging oil to the TIRF objective **[2]**.

4.5.1. Talent at the workbench with the solutions in labeled containers.
4.5.2. Talent adding the imaging oil to the TIRF objective.

4.6. Put the sample chambers on the objective **[1]**. Fill the chamber with 10 microliters of streptavidin solution and wait for 1 minute **[2]**. Wash the chamber with 30 microliters of TAM_{10}_WT buffer, collecting the run-through solution with a folded filter paper. Wait for 1 minute after washing **[3]**.

NOTE: Milliliter was changed with microliter in VO narration for step 4.6 by the authors.

4.6.1. Talent placing the sample chambers on the objective.
4.6.2. Talent filling the chamber with streptavidin solution.
4.6.3. Talent washing the chamber with TAM_{10}_WT buffer.

4.7. Dilute the ribosome complexes, PRE or POST, to 10 to 50 nanomolar concentrations with TAM_{10}_WT buffer **[1]**. Load 20 microliters of the ribosome sample into the channel and wait for 2 minutes **[2]**. Make the imaging buffer using 50 microliters of TAM_{10} buffer and 0.5 microliters each of deoxy, glucose, and Trolox solution. Mix them well **[3].**

NOTE: Milliliter was changed with microliter in VO narration for step 4.7 by the authors.

4.7.1. Talent diluting the ribosomal complexes.
4.7.2. Talent loading the ribosome sample into the channel.
4.7.3. Talent preparing the imaging buffer.

4.8. Flush the chamber with 30 milliliters of the imaging buffer **[1]**. Set the camera for acquiring images **[2]**. Click the upper shutter **On** and the lamp **Off** **[3]**.

4.8.1. Talent flushing the chamber with the imaging buffer.
4.8.2. SCREEN: 62664.4.8.2_TO_4.9.5_T2.mp4. 00:03 – 00:08. *Video Editor: Emphasize the following settings on screen:* ***16 bits, 4 x 4 binning , 100 ms/frame***
4.8.3. SCREEN: 62664.4.8.2_TO_4.9.5_T2.mp4. 00:08 – 00:11

4.9. Start the camera acquisition and spread the images into three windows representing two individual cameras and the overlay **[1]**. Under the **Acquire** menu select **Capture time-lapse** and then click on **Capture Automatically [2]**. Set the appropriate file name. file path and the number of loops and click on **Run now [3]**.*Videographer: This step is important!*

4.9.1. SCREEN: 62664.4.8.2_TO_4.9.5_T2.mp4. 00:12 – 00:22.
4.9.2. SCREEN: 62664.4.8.2_TO_4.9.5_T2.mp4. 00:25 – 00:30.
4.9.3. SCREEN: 62664.4.8.2_TO_4.9.5_T2.mp4. 00:30 – 00:38.

4.10. Close the popup window once the image acquisition is complete **[1]** and then open the acquired ND file **[2]**.

4.10.1. SCREEN: 62664.4.8.2_TO_4.9.5_T2.mp4. 00:40 – 00:50. *Video editor speed up the video from 00:40 till 00:45*
4.10.2. SCREEN: 62664.4.9.6_TO_4.11.3. mp4. 00:03 – 00:08.

4.11. Click on **ROI/simple ROI** editor and choose the option **Circle [1]**. Select the ROI on the image and click on **Finish** when it is completed **[2]**.

4.11.1. SCREEN: 62664.4.9.6_TO_4.11.3.mp4. 00:25 – 00:31
4.11.2. SCREEN: 62664.4.9.6_TO_4.11.3.mp4. 00:33 – 00:47 *Video editor speed up the video from 00:33 till 00:43*

4.12. Click on **Measurement/Time Measurement** to show the data either as a plot or a spreadsheet **[1]**. Open the **Export** tab and set the export parameters, then click on **Export** to save the intensities **[2]**. Finally, open the file which has been exported using a suitable application **[3]**.

4.12.1. SCREEN: 62664.4.9.6_TO_4.11.3.mp4. 00:48 – 00:56 *Video editor speed up the video from 00:52 till 00:56*
4.12.2. SCREEN: 62664.4.9.6_TO_4.11.3.mp4. 01:14 – 01:27 *Video editor speed up the video from 01:22 till 01:27*
4.12.3. SCREEN: 62664.4.9.6_TO_4.11.3.mp4. 01:33 – 01:35, 01:41 – 01:46.

Results

5. Results: Single-Molecule Fluorescence Energy Transfer of Ribosome Protein Synthesis

5.1. The distance from the L27 labeling residue to the A-site or P-site tRNA is 61 or 52 angstrom, respectively, corresponding to FRET efficiency of 0.47 and 0.65 **[1]**. Fluorescence intensities from the donor and acceptor channels were retrieved and plotted as time lapses **[2]**

5.1.1. LAB MEDIA: Figure 1. *Video editors include only the two diagrams at the top.*

5.1.2. LAB MEDIA: Figure 1. *Video editors include only the diagrams at the bottom.*

5.2. After donor bleaching, both traces approached the baseline because no excitation could occur directly on the acceptor **[1]**. After acceptor bleaching, the donor intensity increased because fewer reaction pathways dissipated the excitation energy **[2]**.

5.2.1. LAB MEDIA: Figure 2A. *Video editor include the key for the graph.*

5.2.2. LAB MEDIA: Figure 2B and 2C. *Video editor include the key for the graph.*

5.3. The individual ribosomes exhibited different fluctuations due to the wobbling motion of the tRNAs, which caused distance fluctuations to the L27 **[1].**

5.3.1. LAB MEDIA: Figure 2.

Conclusion

6. Conclusion Interview Statements

6.1. **Yuhong Wang:** Methods like Puromysine and polyPhe assays, Toe-priting assay and Mass Spec are complementary to smFRET and reveal more details about protein translation.

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

6.2. **Yuhong Wang:** smFRET has a broader application because many biological processes occur in the nanometer range and at millisecond time scale. By tagging proper dyes at proper positions, other dynamics can be revealed.

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