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Title: FLIM-FRET Measurements of Protein-Protein Interactions in Live Bacteria

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Yes**

   If **Yes**, can you record movies/images using your own microscope camera?

   Yes

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

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

   If **Yes**, how far apart are the locations? **5-10 min walking distance**

Current Protocol Length

Number of Steps: 17
Number of Shots: 37

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Hanna Manko:** FLIM-FRET is a powerful technique that makes it possible to confirm suspected or predicted PPIs. In this protocol we describe the way to analyze data in particular cases of unbalanced donor/acceptor quantities.

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

1.2. **Hanna Manko:** FLIM-FRET is able to provide information about PPIs directly in live cells. It is important to investigate PPIs in the native cellular environment, in particular when fluorescent proteins are expressed at the endogenous level.

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

Introduction of Demonstrator on Camera

1.3. **Hanna Manko:** Demonstrating the procedure will be Quentin Perraud, a PhD student from Dr Isabelle Schalk's laboratory.

1.3.1. INTERVIEW: Author saying the above.

1.3.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Protocol

2. Fluorescent Tag Insertion into the Chromosomal Genome of P. aeruginosa

2.1. Begin by growing *P. aeruginosa*, *E. coli* TOP10, and *E. coli* helper bacteria, each in 5 milliliters of LB without antibiotic, at 30 degrees Celsius while shaking overnight **[1]**. On the next day, measure the optical density of the cultures **[2]** and mix an equal quantity of *P. aeruginosa*, *E. coli* TOP10, and *E. coli* helper in a 1.5-milliliter microtube **[3-TXT]**.

2.1.1. WIDE: Establishing shot of talent placing culture tubes in the incubator and closing the lid.

2.1.2. Talent measuring OD600 of one of the cultures.

2.1.3. Talent adding the cultures to a microtube. **TEXT: 500 µL each, $OD_{600\ nm}$ = 1.0**

2.2. Centrifuge the tube for 5 minutes at 9,300 x *g* to pellet the bacteria **[1]**, then discard the supernatant **[2]** and resuspend the pellet in 50 microliters of LB **[3]**. Plate a spot of the mixture on preheated LB agar and incubate it for 5 hours at 37 degrees Celsius **[3]**.

2.2.1. Talent putting the tube in the centrifuge and closing the lid.

2.2.2. Talent removing the supernatant.

2.2.3. Talent resuspending the pellet.

2.2.4. Talent plating a spot of the mixture on LB agar.

2.3. After the incubation, scrape the spot with a sterile inoculation loop **[1]** and resuspend it in 1 milliliter of LB **[2]**. Plate 100 microliters of this bacterial suspension on LB agar containing 10 micrograms per milliliter chloramphenicol and 30 micrograms per milliliter gentamicin **[3]**, then incubate the plate for 2 days at 37 degrees Celsius **[4]**. *Videographer: This step is important!*

2.3.1. Talent scraping the spot.

2.3.2. Talent resuspending the bacteria.

2.3.3. Talent plating the suspension.

2.3.4. Talent putting the plates in the incubator and closing the door.

2.4. Resuspend one colony in 1 milliliter of LB **[1]** and incubate it at 37 degrees Celsius with orbital shaking for 4 hours **[2]**. Then, centrifuge the tube for 3 minutes at 9,300 x *g* **[3]** and discard 950 microliters of supernatant **[4]**.

2.4.1. Talent transferring a colony from the plate to the LB.

2.4.2. Talent putting the LB tube in the incubator and starting the shaking.

2.4.3. Talent putting the tube in the centrifuge and closing the lid.

2.4.4. Talent removing the supernatant.

2.5. Resuspend the pellet in 50 microliters of LB **[1]** and isolate the mixture on LB agar containing sucrose and no sodium chloride **[2]**. Incubate the plate overnight at 30 degrees Celsius **[3]**.

2.5.1. Talent resuspending the pellet in LB.

2.5.2. Talent plating the mixture on the agar.

2.5.3. Talent putting the plate in the incubator and closing the door.

2.6. Spot isolated colonies on LB agar and LB agar containing 15 milligram per milliliter gentamicin in order to check for gentamicin sensitivity **[1]**. *Videographer: This step is important!*

2.6.1. Talent spotting colonies on agar plates.

3. Preparation of Agarose Pad

3.1. Place a microscope glass slide on a flat horizontal surface and arrange two glass slides topped with two layers of adhesive tape around it **[1]**. Pipette a 70-microliter droplet of 1% melted agarose onto the glass slide **[2]** and place a fourth slide on top to flatten the agarose droplet. Press down gently for about a minute **[3]**.

3.1.1. Talent putting down the slide and putting the other 2 slides on each side.

3.1.2. Talent pipetting agarose on the slide.

3.1.3. Talent covering the agarose with another slide and gently pressing down.

3.2. Take off the upper slide and drop about 3 microliters of bacteria in 3 to 4 spots at different locations on the agarose pad **[1]**. Cover the agarose pad with a microscopy glass coverslip **[2]** and fix it with melted paraffin to seal it onto the glass slide, starting with the four corners **[3]**.

3.2.1. Talent taking off the slide and dropping bacteria on the pad.

3.2.2. Talent covering the agarose pad with a coverslip.

3.2.3. Talent fixing the coverslip with melted paraffin starting by fixing the corners.

4. Imaging with a Two-photon Microscopy Setup

4.1. Place the microscopy slide on the stage with the coverslip facing the objective **[1]**. Turn the filter cube turret to select the eGFP cube and open the fluorescence lamp shutter, then send the fluorescence light towards the eyepiece of the microscope **[2]**. Focus the objective on the bacteria using the microscope knob **[3]**.

4.1.1. Talent positioning the slide on the stage.

4.1.2. Talent turning the filter cube turret, opening the fluorescence lamp shutter, then sending the fluorescence light towards the eyepiece of the microscope.

4.1.3. Talent using the knob to focus.

4.2. Select a region of interest in the sample using the joystick that controls the motorized stage **[1]**. Send the fluorescence emission path back towards the detector. Then, turn back the filter cube turret to select the dichroic cube for the 930-nanometer laser **[2]** and set the laser power to 20 milliwatts **[3]**.

4.2.1. Talent selecting the region of interest.

4.2.2. Talent sending the fluorescence emission towards the detector and turning the filter cube turret.

4.2.3. Talent setting the laser power in the software.

4.3. Set the size of the region of interest to 30 micrometers, which adjusts the voltage operating the galvo-mirrors and defines the range of their movements **[1]**. Turn on the detector **[2]** and start scanning the sample **[3]**.

4.3.1. SCREEN: 61602_screenshot_1. 0:03 – 0:10. Region size set.

4.3.2. Talent turning on the detector.

4.3.3. SCREEN: 61602_screenshot_1. 0:10 – 0:17. Scanning started.

4.4. Choose the field of view for imaging by finely moving the stage from the computer interface. This can be done on the setup by moving the cross on the image in the microscope control software, which will define the new center of the image, and pressing **Move Stage [1]**.

4.4.1. SCREEN: 61602_screenshot_1. 0:18 – 0:26. Field of view being selected.

4.5. A good field of view for acquisition should contain 10 to 30 immobile bacteria, all in focus. If interested in extracting single cells FLIM-FRET data, ensure that the bacteria are well individualized **[1]**.

4.5.1. SCREEN: 61602_screenshot_1. 0:26 – 0:30. Good field of view.

4.6. Open the imaging software and check that the photon count rate is not too high to avoid a pile-up effect that can influence lifetime measurements. If necessary, lower the laser intensity to keep the photon count rate low **[1]**.

4.6.1. SCREEN: 61602_screenshot_2. 0:05 – 0:10. SPCM software opened and photons count rate checked.

4.7. Adjust acquisition parameters, including the acquisition collection time, press the **Start** button, and wait for the acquisition to complete **[1]**.

4.7.1. SCREEN: 61602_screenshot_2. 0:10 – 0:32. Parameters adjusted and Start pressed.

4.8. To analyze the data, open RStudio and create a new project **[1]**. Create a new folder in the main project folder and name it "data" **[2]**, then move all analysis *asc* files into this folder **[3]**. Open a new script file or the supplementary script FLIM-underscore-analysis-dot-R **[4]**.

4.8.1. SCREEN: 61602_screenshot_3. RStudio opened, new project created.

4.8.2. SCREEN: 61602_screenshot_4. Data folder created.

4.8.3. SCREEN: 61602_screenshot_5. Files moved into appropriate folder.

4.8.4. SCREEN: 61602_screenshot_6. New script file opened.

4.9. Install the dedicated filmDiagRam package for film data analysis and call the package in the workspace **[1-TXT]**.

4.9.1. Talent at the computer installing a package and calling it in the workspace.
TEXT: https://github.com/jgodet/flimDiagRam ; HowTo_FlimDiagRam
Tutorial at https://github.com/jgodet/flimDiagRam/blob/master/HowTo.pdf

Results

5. Results: FLIM-FRET Data Visualization and Analysis

5.1. Empirical cumulative distribution functions of the fluorescence lifetimes measured for the different bacterial strains are shown here **[1]**. If FRET occurs, the functions are shifted towards the shorter-lived lifetimes **[2]**.

5.1.1. LAB MEDIA: Figure 8.

5.1.2. LAB MEDIA: Figure 8. *Video Editor: Emphasize A and B.*

5.2. It is important to note that FRET can't occur when the interaction of the two proteins results in a long distance between the two fluorophores, which can't be distinguished from the absence of interaction. Therefore, it may be necessary to label the proteins at different positions to maximize the chances of probing the interaction **[1]**.

5.2.1. LAB MEDIA: Figure 8. *Video Editor: Emphasize C.*

5.3. Due to the large difference in protein expressions between PvdA and PvdL, the same complex does not result in similar FLIM-FRET data. Unbalanced stoichiometries lead to differences in the contribution of the free as compared to the bound donor-labelled proteins in the recorded fluorescence lifetime distribution **[1]**.

5.3.1. LAB MEDIA: Figure 8 A and B.

5.4. The diagram plot can be used to provide critical information about the stoichiometry **[1]**. In the PvdA-eGFP-mCherry PvdL mutant, the quantity of donor-labelled PvdA is much higher than the quantity of mCherry PvdL **[2]**.

5.4.1. LAB MEDIA: Figure 9.

5.4.2. LAB MEDIA: Figure 9 A.

5.5. Amongst all donors present in the sample, only a few of them are interacting with PvdL **[1]**. The single tau1 value centered at approximately 2.3 nanoseconds suggests that each PvdA-eGFP donor can only transfer with one mCherry PvdL acceptor **[2]**.

5.5.1. LAB MEDIA: Figure 9 A.

5.5.2. LAB MEDIA: Figure 9 A. *Video Editor: Emphasize the area enclosed in red.*

5.6. When the labelling is reversed, most of the eGFP PvdL proteins are expected to interact with PvdA-mCherry **[1]**, which is confirmed by the higher alpha1 values. The tau1 values became more distributed, suggesting that multiple PvdA proteins may bind to a single PvdL protein **[2]**.

5.6.1. LAB MEDIA: Figure 9 B.

5.6.2. LAB MEDIA: Figure 9 B. *Video Editor: Emphasize the area enclosed in red.*

Conclusion

6. Conclusion Interview Statements

6.1. **Hanna Manko:** The FLIM setup is becoming available in an increasing number of imaging facilities. Imaging samples in FLIM-FRET is no more complicated than imaging in video microscopy.

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