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Title: Monitoring neutrophil elastase and cathepsin G activity in human sputum samples

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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? **NO**

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?

   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? **NO**

Current Protocol Length

Number of Steps: 20
Number of Shots: 48

Introduction

1. **Introductory Interview Statements**

REQUIRED:

1.1. **Dario L Frey:** Our FRET probes and protocols enable rapid and sensitive quantification of free and surface-bound elastase and cathepsin G activity in sputum from patients with neutrophilic airway diseases.

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

1.2. **Matteo Guerra:** These methods make it possible to explore different aspects of proteases pathophysiology. Plate reader measurements permit for large screenings, confocal microscopy visualizes enzyme activity with subcellular resolution while flow cytometry enables single-cell phenotyping and protease activity quantification in a personalized fashion.

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

OPTIONAL:

1.3. **Dario L Frey:** This technique can be expanded to different airway diseases such as CF, bronchiectasis or COPD and it can be adapted to different biosamples such as blood and bronchoalveolar lavages or mice samples.

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

Ethics Title Card

1.4. The following protocols describe analysis performed on human sputum. Human sample handling was approved by the ethics committee of the University of Heidelberg.

Protocol

2. Cell isolation and supernatant separation

2.1. Before starting the sputum induction procedure, inhale 200 micrograms of the β-2-receptor-antagonist (*Pronounce 'Beta-Two')* salbutamol **[1]**. Afterwards, inhale hypertonic 6 percent saline solution for 15 minutes using a nebulizer **[2]**. Collect the expectorated sputum in a Petri dish **[3]**.

2.1.1. Talent starting the sputum induction procedure by inhaling 200 micrograms of the β-2-receptor-antagonist salbutamol.
2.1.2. Talent inhaling hypertonic 6% saline solution for 15 minutes using a nebulizer.
2.1.3. Collection of expectorated sputum in a Petri dish.

2.2. Separate mucus clumps from the saliva into a Petri dish with the help of a pipette tip **[1]**. Weigh the mucus **[2]**, then add 4 volume parts per weight of 10 percent Sputolysin in PBS to the sputum **[3-TXT].**

2.2.1. Talent separating the mucus clumps from the saliva.
2.2.2. Talent measuring weight of mucus.
2.2.3. Talent adding 10% Sputolysin to sputum**. TEXT: 4 mL of 10% Sputolysin for each gram of sputum**

2.3. Incubate the mixture at room temperature on a rocking shaker for 15 minutes to dissolve the mucus **[1-TXT]**. Quench the reaction by adding 1 milliliter of cold PBS for each milliliter of 10 percent Sputolysin **[2]**. Pipette the mixture to obtain a homogenous solution **[3]**.

2.3.1. Talent putting the mixture for incubation on rocking shaker. **TEXT: Caution: Work in fume hood**
2.3.2. Talent quenching the reaction by adding PBS.
2.3.3. Talent mixing the reaction to get homogenous solution.

2.4. Filter the mixture through a 100-micron nylon cell strainer into a 50-milliliter tube **[1]**. Repeat the filtration step through a 40-micron strainer **[2]**, then centrifuge for 10 minutes at 300 x g at 4 degrees Celsius **[3]**. Transfer the supernatant into a fresh tube and store it on ice **[4]**.

2.4.1. Talent filtering the mixture through a 100-micron nylon cell strainer.
2.4.2. Talent filtering the mixture through a 40-micron filter.

2.4.3. Talent putting the tubes in the centrifuge.
2.4.4. Talent transferring the supernatant fraction to fresh tube on ice.

2.5. Gently resuspend the cell pellet in 500 microliters of cold PBS and place it on ice **[1]**.

2.5.1. Talent resuspending the pellets in 500 microliters of cold PBS.

3. Quantification of soluble neutrophil serine protease (NSP) activity via fluorimeter or plate reader assay

3.1. Thaw enzymes on ice **[1]** and set up an enzyme standard curve as described in the text manuscript **[2]**. In parallel to the standard preparation, dilute sputum samples in activation buffer **[3]**. *Videographer: This step is difficult!*

3.1.1. Talent keeping the enzymes on ice for thawing.
3.1.2. Talent setting an enzyme standard curve.
3.1.3. Talent diluting the sputum samples.

3.2. On the plate reader, set the excitation wavelength **[1-added]** for the NE FRET probe NEmo-1 *(Pronounce 'nemo-one')* to 354 nanometers and the emission wavelength to 400 nanometers for donor and 490 nanometers for acceptor. For the CG FRET probe sSAM, *(Pronounce 'ɛs/[SAM]' SAM is pronounced like in 'sample)* set the excitation wavelength to 405 nanometers and emission to 485 nanometers for donor and 580 nanometers for acceptor **[2]**.

3.2.1. Added shot: Talent arriving at the instrument/computer
3.2.2. Talent setting up the wavelength on the instrument for the detection of probes.

3.3. Add 40-microliters of samples, standards, or blanks into the wells of a black 96-well half area plate **[1]** and add the master mix **[2-TXT]**. Start the plate reader and record the donor to acceptor ratio increase after every 60 to 90 seconds for at least 20 minutes or until the increase in the signal reaches a plateau **[3]**.

3.3.1. Talent adding the samples to a 96-well plate.
3.3.2. Talent adding master mix. **TEXT: 10X number of plate wells.**
3.3.3. Talent recording the donor/acceptor ratio increase every 60-90 seconds.

3.4. Export the data and calculate the donor to acceptor ratio by dividing donor RFU with the acceptor RFU for each time point and sample. Then, calculate the donor to acceptor

ratio mean and standard deviation. Determine the slope within the linear growth of the donor to acceptor ratio change **[1]**.

3.4.1. SCREEN: Data exported, donor to acceptor ratios calculated, mean and standard deviation calculated, and slope determined. *Videographer: Film the screen here.*

4. Membrane-bound NSPs activity measurement via fluorescence microscopy

4.1. For each measurement, resuspend thirty thousand sputum cells in a volume of 50 microliters of PBS in a 1.5-milliliter tube.

4.1.1. Talent resuspending the described number of cells in PBS.

4.2. Incubate the sputum cells with a specific inhibitor **[1]** as a negative control and an appropriate enzyme as a positive control for 10 minutes at room temperature **[2]**. *Videographer: This step is difficult and important!*

4.2.1. Talent setting up the negative control.
4.2.2. Talent setting up the positive control.

4.3. Add 50-microliters of PBS containing FRET reporter and a nuclear stain to each tube to get a final concentration of 2 micromolar **[1-TXT]**. Incubate the mixture at room temperature for 10 to 20 minutes **[2]**. *Videographer: This step is important!*

4.3.1. Talent adding FRET reporter and nuclear stain to a few tubes. **TEXT: Final dilution 1:1000.**
4.3.2. Talent placing the samples on lab bench for incubation.

4.4. Quench the reaction by adding 100-microliters of ice-cold PBS **[1]** and place the samples on ice **[2]**. Cytospin the mixture on microscopy slides **[3]**, then air dry and fix the cells with ice cold 10 percent methanol for 10 minutes **[4]**. After fixation, air dry and mount the sample with an appropriate mounting medium **[5]**.

4.4.1. Talent adding PBS to the reaction mixture.
4.4.2. Talent placing the samples on ice.
4.4.3. Talent using the cytocentrifuge.
4.4.4. Talent adding methanol to the sample.
4.4.5. Talent mounting the sample on a slide.

4.5. Capture the images using a confocal microscope. Image at least 100 cells per condition for conclusive statistics **[1-TXT]**.

4.5.1. Talent acquiring the images. **TEXT: PL APO 40x or 63x oil objective**

5. Membrane-bound NSPs activity measurement via flow cytometry

5.1. Resuspend 1 million cells in 100-microliters of PBS in a 5-milliliter FACS polystyrene round-bottom tube and place the tube on ice **[1]**. Add 2-microliters of FcBlock to each sample **[2]**, then incubate the samples for 5 minutes at room temperature **[3]**.

5.1.1. Talent resuspending the cells in PBS.
5.1.2. Talent adding FcBlock to each sample.
5.1.3. Talent placing the samples on lab bench for incubation.

5.2. Add antibody to each tube **[1]** and incubate on ice in the dark for 30 minutes **[2].** Wash the cells with 2 milliliters of cold PBS and centrifuge at 300 x g and 4 degrees Celsius **[3].** Finally, resuspend in 200 microliters of PBS **[4]**.

5.2.1. Talent adding antibody to a few tubes.
5.2.2. Talent placing the samples for incubation in dark.
5.2.3. Talent giving the washes with PBS.
5.2.4. After centrifugation, talent resuspending the cells in PBS.

5.3. Divide the suspension into two tubes, 100-microliters each, and add 5-microliters of cell viability stain **[1]**. Add appropriate specific NSP inhibitor to the negative control tube **[2]** and incubate the sample for 10 minutes at room temperature in the dark **[3]**. *Videographer: This step is difficult and important!*

5.3.1. Talent dividing the sample and adding stain.
5.3.2. Talent adding the NSP inhibitor.
5.3.3. Talent placing the samples in dark for incubation.

5.4. Add PBS to the sample and filter it through a 40-micron filter into a clean FACS tube **[1]**.

5.4.1. Talent filtering the sample through a filter.

5.5. Add the reporter to the negative control sample and gently vortex **[1]**. Start acquiring cells incubated with the specific inhibitor and, if necessary, slightly adjust the gates as well as the reporter PMTs' voltages **[2]**. *Videographer: This step is important!*

5.5.1. Talent adding the reporter stain.
5.5.2. Talent starting acquisition.

5.6. To record changes in the donor to acceptor ratio due to membrane-bound protease activity, record 1000 neutrophils from each tube every 5 to 10 minutes **[1]**. Calculate the FRET ratio by dividing the donor by the acceptor channel values for the samples measured on the gated viable single neutrophils **[2]**. *Videographer: This step is important!*

5.6.1. Talent recording the changes in D-A ratio.
5.6.2. Talent calculating the FRET ratio.

Results

6. Results: Representative images and quantification of membrane-bound NE activity on neutrophils isolated from CF patient sputum

6.1. Images of neutrophils pre-incubated with 100-micromolar Sivelestat **[1]** or left untreated before reporter NEmo-2 addition are shown here **[2]**.

6.1.1. LAB MEDIA: Figure 1a. *Video Editor: Emphasize on top panel.*
6.1.2. LAB MEDIA: Figure 1a. *Video Editor: Emphasize on bottom panel.*

6.2. Nuclear signal was used to identify neutrophils by their characteristic segmented nuclei and the ROI was selected manually **[1]**. The donor to acceptor ratio of sputum neutrophils was plotted. Each dot represents the mean of one ROI **[2]**.

6.2.1. LAB MEDIA: Figure 1a. *Video Editor: Emphasize the dashed line in figure.*
6.2.2. LAB MEDIA: Figure 1b.

6.3. Flow cytometry gating makes it possible to discriminate and study sputum neutrophils **[1]**. The MFI distribution at 0 and 10 minutes was observed after reporter addition **[2]**. The mean donor and acceptor MFI values for 1000 sputum neutrophils were calculated **[3]**. The D-A ration reached a plateau after an initial increase **[4]**.

6.3.1. LAB MEDIA: Figure 2a.
6.3.2. LAB MEDIA: Figure 2b.
6.3.3. LAB MEDIA: Figure 2c. *Video Editor: Emphasize on row values in table.*
6.3.4. LAB MEDIA: Figure 2c. *Video Editor: Emphasize on the right-side of graph.*

Conclusion

7. Conclusion Interview Statements

7.1. **Dario L Frey:** The healthy donor sputum induction may take some practice before perfection. Remember to relax and to breathe the aerosol for 10 to 15 minutes before expectoration. In addition, always keep sputum cells on ice and process them as soon as possible after expectoration.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.1.*

7.2. **Matteo Guerra:** Surface-bound protease activity correlates with early lung damage in children and severity of lung disease in adult CF patients. These methods enable the exploration of proteases as early inflammation biomarkers in neutrophilic airway diseases.

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