FINAL SCRIPT: APPROVED FOR FILMING

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Title: Enzymatic Modification and Flow Cytometry Assessment of Yeast Surface Displayed Proteins

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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**

3. **Filming location:** Will the filming need to take place in multiple locations? **NO**

Current Protocol Length
Number of Steps: 34
Number of Shots: 54

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

REQUIRED:

1.1. **Alexandra Tang:** This research focuses on studying the chemical modifications to proteins that dictate how cells behave. In this case, the goal is to understand the preferences and kinetics of enzymes modifying a variety of substrates.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 2B, 2C, 2D.*

What significant findings have you established in your field?

1.2. **Jose Ezagui:** The ability to dephosphorylate a substrate molecule displayed on the surface of yeast has been demonstrated for the first time. This finding opens possibilities for generalizable kinetic mapping of enzymatic modifications that were previously not accessible.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 3.*

What research gap are you addressing with your protocol?

1.3. **Jose Ezagui:** This research addresses the challenges associated with understanding enzyme-substrate interactions. Typically, cell lysates or customized peptides are required for this type of assessment. Here, a facile alternative is provided through the use of yeast-displayed substrates.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.4.1.*

How will your findings advance research in your field?

1.4. **Alexandra Tang:** This protocol advances traditional yeast surface display towards analysis of what would ordinarily be intracellular enzymatic reactions in the extracellular space, enabling facile analysis of these normally difficult-to-assess interactions.

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

What new scientific questions have your results paved the way for?

1.5. **Lawrence A. Stern:** This protocol enables the ability to interrogate substrate preferences of a variety of enzymes through high-throughput methods and to perform kinetic studies with a simple readout.

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

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Cell Growth of Yeast Harboring Plasmid and Induction of Protein Expression

Demonstrator: Alexandra Tang

2.1. To begin, thaw the prepared one aliquot per plasmid on ice **[1]**. Add 0.5 to 1.5 micrograms of plasmid DNA containing the yeast display construct directly to the cells **[2]**. Then, add 0.5 milliliters of either the transformation solution or a sterile 50 percent polyethylene glycol and 0.1 molar lithium acetate solution **[3]**. Thoroughly combine the mixture of cells, plasmid DNA, and transformation solution by pipetting **[4]**.

2.1.1. WIDE: Talent thawing the prepared plasmid aliquots on ice.

2.1.2. Talent adding plasmid DNA containing the yeast display construct to the cells.

2.1.3. Talent adding transformation solution or polyethylene glycol and LiOAc solution to the mixture.

2.1.4. Talent pipetting the mixture up and down to mix.

2.2. Incubate the transformation mixture statically for 30 to 60 minutes at 30 degrees Celsius **[1]** and vortex the mixture at 15-minute intervals **[2]**. Harvest the cells by centrifuging at 1,000 *g* **[3]**.

2.2.1. Talent placing transformation mixture in the incubator.

2.2.2. Talent vortexing the mixture.

2.2.3. Talent centrifuging the transformation mixture.

2.3. Prepare a 14-milliliter culture tube with 4.5 milliliters of plasmid-containing yeast cell growth or SD-CAA *(S-D C-A-A)* media **[1]**. Resuspend the cells containing the desired plasmid in 500 microliters of SD-CAA **[2]** and inoculate the prepared 4.5 milliliters of media **[3]**.

2.3.1. Talent pouring 4.5 milliliters of SD-CAA media into a 14-milliliter culture tube.

2.3.2. Talent resuspending cells in 500 microliters of SD-CAA.

2.3.3. Talent inoculating the SD-CAA media.

2.4. Distribute 50 microliters of the 5 milliliters of inoculated culture onto a SD-CAA plate

carefully without piercing the agar **[1]**. Incubate statically at 30 degrees Celsius for 48 hours to determine transformation efficiency **[2]**.

2.4.1. Talent pipetting the inoculated SD-CAA culture onto the plate without damaging agar.

2.4.2. Talent placing the plate into the incubator.

2.5. Now, incubate the 5 milliliters of SD-CAA cell culture in a shaking incubator at 30 degrees Celsius and 300 rpm for at least 18 hours **[1]**. Monitor optical density at 600 nanometers after 16 hours and repeat again after 20 hours **[2]**. Once the sample reaches an optical density not exceeding 6, centrifuge the culture for 3 minutes at 2,500 *g* **[3]** and discard the supernatant without disturbing the yeast pellet **[4]**.

2.5.1. Talent placing culture in a shaking incubator.

2.5.2. Talent measuring optical density at 600 nanometers at 16 hours. *Videographer: If possible, try to capture both the talent (monitoring the optical density) and the computer screen (the optical density being calculated) together in the frame. Make sure the computer screen is clearly visible.*

2.5.3. Talent centrifuging culture.

2.5.4. Talent discarding supernatant.

2.6. Resuspend the yeast pellet in the induction of protein expression or SRG-CAA media to a final optical density at 600 nanometers less than 1 **[1-TXT]**.

2.6.1. Talent resuspending yeast pellet in SRG-CAA. **TXT: OD_{600nm} <1 corresponds to <1 × 10^7 yeast/mL**

2.7. Incubate the yeast culture in a shaking incubator at 30 degrees Celsius and 300 rpm for at least 8 hours but no longer than 24 hours **[1]**.

2.7.1. Talent placing yeast culture in a shaking incubator.

2.8. Then, measure the optical density at 600 nanometers to determine the cell density **[1]**.

2.8.1. Talent measuring optical density using a spectrophotometer.

3. Dephosphorylation of Substrates Expressed on the Yeast Cell Surface

Demonstrator: Jose Ezagui

3.1. Prepare the working buffer for the samples by diluting the 2X *(two-ex)* working buffer solution in a 1:2 *(one to two)* ratio with deionized water in a 1.7-milliliter vial **[1]**.

3.1.1. Talent diluting 2X buffer solution with deionized water in a 1.7-milliliter vial.

3.2. Add the appropriate volume of yeast culture required to recover two million yeast cells into the 1.7-milliliter vial for each sample **[1-TXT]**.

3.2.1. Talent adding yeast culture to the 1.7-milliliter vial. **TXT: Calculate the volume of yeast culture based on the measured optical density**

3.3. Centrifuge the vial for 1 minute at 4,500 *g* **[1]**, and using a micropipette, carefully remove and discard the supernatant as biohazardous waste **[2]**.

3.3.1. Talent centrifuging the vial.

3.3.2. Talent discarding the supernatant.

3.4. Resuspend the pelleted cells in 1 milliliter of PBSA *(P-B-S-A)* before repeating the centrifugation and supernatant removal. Keep it aside **[1-TXT]**.

3.4.1. Talent resuspending the pelleted cells in 1 milliliter of PBSA. **TXT: PBSA: PBS with 1 g/L bovine serum albumin**

3.5. Prepare the recombinant human SHP-2 *(S-H-P two)* solution at a final concentration of 1,000 nanomoles in a 20-microliter total reaction volume **[1-TXT]**.

3.5.1. A shot of the recombinant human SHP-2 solution with a final concentration of 1,000 nanomoles in a 20 microliter total reaction volume. **TXT: SHP-2: Src homology region 2 domain-containing phosphatase-2**

3.6. Next, add 7.7 milligrams of DTT *(D-T-T)* into 10 milliliters of deionized water in a 15-milliliter conical tube to create a 5-millimolar DTT solution **[1]**.

3.6.1. Talent preparing 5 millimolar DTT solution in a 15-milliliter conical tube.

3.7. Now, take the pelleted cells and resuspend them in the working buffer so that the final reaction volume is 20 microliters per sample **[1]**.

3.7.1. Talent resuspending cells in working buffer.

3.8. Add 2 microliters of the 5 millimolar DTT solution to each sample for a final DTT concentration of 0.5 millimolar **[1]**.

3.8.1. Talent adding DTT to samples.

3.9. Add the prepared volume of SHP-2 to each sample to achieve a final volume of 20 microliters and gently mix using a micropipette **[1]**.

3.9.1. Talent adding SHP-2 to sample and mixing.

3.10. Wrap the sample vial lids with parafilm to prevent leakage or cross-contamination **[1]**.

3.10.1. Talent wrapping vials with parafilm.

3.11. Incubate the samples at 37 degrees Celsius for 2 hours on a rotor at a constant speed **[1]**.

3.11.1. Talent placing vials on rotor.

3.12. After removing the samples from the rotor, stop the reaction by adding 1 milliliter of PBSA to each sample before centrifuging them again for 1 minute **[1]**. Then, discard the supernatant **[2]**.

3.12.1. Talent adding PBSA to stop the reaction.

3.12.2. Talent discarding the supernatant after centrifugation.

4. Cell Labeling and Flow Cytometry Analysis of Dephosphorylated Substrates
Demonstrator: Alexandra Tang and Jose Ezagui

4.1. Resuspend the pelleted samples in a 20-microliter mix of their corresponding primary

reagents and incubate for 20 minutes at room temperature **[1]**.

4.1.1. Talent adding the 20-microliter mix of the corresponding primary reagent to the pellet.

4.2. After 20 minutes, centrifuge samples at 4,500 *g* for 1 minute **[1]** and discard the supernatant as biohazardous waste **[2]**.

4.2.1. Talent centrifuging the sample.

4.2.2. Talent discarding supernatant.

4.3. Wash the cells once by resuspending them in 1 milliliter of PBSA before centrifuging the cells again **[1]**.

4.3.1. Talent washing the cells.

4.4. Now, resuspend the samples in a 20-microliter mix of their corresponding secondary reagents **[1]** and incubate for 15 minutes in the dark **[2]**.

4.4.1. Talent adding secondary reagents.

4.4.2. Talent incubating in the dark.

4.5. After centrifuging the samples and discarding the supernatant, wash the cells once more with PBSA before centrifuging them again **[1]**.

4.5.1. Talent washing the cells with PBSA.

4.6. Next, resuspend the washed samples in 300 to 500 microliters of PBSA **[1]** and transfer them to 5-milliliter polystyrene tubes for flow cytometry analysis **[2]**.

4.6.1. Talent resuspending the washed sample in PBSA

4.6.2. Talent transferring the sample into 5-milliliter polystyrene tube.

4.7. After preparing the cytometer, click on **File**, followed by the **New Experiment** button, name the experiment, and click **Save** to ensure the data acquired is saved in the desired file path **[1]**.

4.7.1. SCREEN: 68208_Screenshot_1.mp4 00:01-00:06.

4.8. Select the **dot-plot** icon in the upper toolbar to create two or more dot-plots for each sample. For one of the dot-plots, ensure the X-axis name displays the FSC-A *(F-S-C A)* channel and the Y-axis name displays the SSC-A *(S-S-C A)* channel **[1]**. NOTE: The VO has been edited.

4.8.1. SCREEN: 68208_Screenshot_1.mp4 00:07-00:15.

4.9. For another plot, select the X-axis name to display the channel in which the secondary reagent targeting the primary anti-epitope tag antibody fluoresces, and the Y-axis name to display the channel in which the streptavidin secondary reagent fluoresces **[1]**. Provide a descriptive name for the sample by right-clicking on the tube, selecting **Edit Name**, and entering the sample name **[2]**. NOTE: The VO has been edited.

4.9.1. SCREEN: 68208_Screenshot_1.mp4 00:16-00:28.

Added shot: SCREEN: 68208_Screenshot_1.mp4 00:29-00:35.

4.10. Place each sample tube in the holder of the cytometer **[1]** and click **Run** to begin loading the sample and acquiring data **[2]**. Adjust events, time, and flow rate as necessary **[2]**.

4.10.1. Talent placing the sample tube in the holder of the cytometer.

4.10.2. SCREEN: 68208_Screenshot_2.mp4 00:01-00:03.

4.10.3. SCREEN: 68208_Screenshot_2.mp4 00:04-00:17.

4.11. Define a gate surrounding the healthy yeast cells in the SSC-A versus FSC-A plot **[1]**.

4.11.1. SCREEN: 68208_Screenshot_2.mp4 00:38-00:53.

4.12. Then, record the fluorescence of all control samples **[1]**. Define a gating strategy for the created plot before analyzing the treated samples **[2]**.

4.12.1. SCREEN: 68208_Screenshot_2.mp4 00:54-01:02.

4.12.2. LAB MEDIA: 68208_Screenshot_3.mp4 00:01-00:13.

4.13. Record the fluorescence of dephosphorylated samples using the cytometer and the gating strategy before analyzing the data using flow cytometry software **[1]**.

4.13.1. SCREEN: 68208_Screenshot_3.mp4 00:53-01:03.

4.14. Evaluate dephosphorylation by measuring and comparing the Y-axis median of cells expressing protein on their surface and the baseline phosphorylation provided by non-displaying cells between samples and controls **[1]**. Finally, calculate the percent median phosphorylation difference using the provided formula **[2]**.

4.14.1. SCREEN: 68208_Screenshot_4.mp4 00:01-00:22.

4.14.2. LAB MEDIA: Figure 1A, 1B.

AND,

TEXT on PLAIN BACKGROUND:

$$\%\ \text{Median phosphorylation difference} = \frac{\text{Ymedian(Fig1B, Green)} - \text{Ymedian(Fig1B, Grey)}}{\text{Ymedian(Fig1A, Green)} - \text{Ymedian(Fig1A, Grey)}}$$

Results

5. Results

5.1. The effect of varying SHP-2 phosphatase concentrations and incubation times on the median phosphorylation difference in yeast surface-displayed substrates is illustrated in this figure **[1]**.

5.1.1. LAB MEDIA: Figure 3.

5.2. Percent median phosphorylation difference decreased over time across all SHP-2 concentrations **[1]**, with the highest dephosphorylation observed at 1000 nanomolar SHP-2 concentration at 4 hours **[2].**

5.2.1. LAB MEDIA: Figure 3. *Video Editor: Highlight all bar graphs.*

5.2.2. LAB MEDIA: Figure 3. *Video Editor: Highlight the last red bar on the right (4 hour).*

5.3. After 2 hours of incubation, 1,000 nanomolar SHP-2 resulted in approximately 48.8 percent median phosphorylation difference **[1]**, significantly lower than the 750-nanomolar condition at the same time point **[2]**.

5.3.1. LAB MEDIA: Figure 3. *Video Editor: Highlight the red bar in 2 hour.*

5.3.2. LAB MEDIA: Figure 3. *Video Editor: Highlight the yellow bar in 2 hour.*

5.4. No significant difference in median phosphorylation difference was observed when incubation was extended from 2 hours to 3 hours across 500 nanomolar, 750 nanomolar, and 1,000 nanomolar SHP-2 concentrations **[1]**.

5.4.1. LAB MEDIA: Figure 3. *Video Editor: Highlight the brown, yellow, and red bars in 3 hour.*

5.5. At 4 hours of incubation, a significant decrease in median phosphorylation difference was seen, especially at a 1,000 nanomolar SHP-2 concentration **[1]**.

5.5.1. LAB MEDIA: Figure 3. *Video Editor: Highlight the red bar in 4 hour.*

Pronunciation Guides:

1. Aliquot
Pronunciation link: https://www.merriam-webster.com/dictionary/aliquot
IPA: /ˈælɪˌkwɑt/
Phonetic Spelling: al-ih-kwaat

2. Plasmid
Pronunciation link: https://www.merriam-webster.com/dictionary/plasmid

IPA: /ˈplæz.mɪd/
Phonetic Spelling: plaz-mid

3. Polyethylene Glycol
Pronunciation link: https://www.howtopronounce.com/polyethylene-glycol
IPA: /ˌpɑliˈɛθəˌlin ˈglaɪˌkɔl/
Phonetic Spelling: pah-lee-eth-uh-leen gly-kawl

4. Lithium Acetate
Pronunciation link: https://www.howtopronounce.com/lithium-acetate
IPA: /ˈlɪθiəm ˈæsəˌteɪt/
Phonetic Spelling: lith-ee-um ass-uh-tayt

5. Centrifuge
Pronunciation link: https://www.merriam-webster.com/dictionary/centrifuge
IPA: /ˈsɛntrəˌfjuːdʒ/
Phonetic Spelling: sen-truh-fyooj

6. Inoculate
Pronunciation link: https://www.merriam-webster.com/dictionary/inoculate
IPA: /ɪˈnɑkjəˌleɪt/
Phonetic Spelling: ih-nahk-yuh-layt

7. Supernatant
Pronunciation link: https://www.merriam-webster.com/dictionary/supernatant
IPA: /ˈsuːpərˌneɪtənt/
Phonetic Spelling: soo-per-nay-tuhnt

8. Spectrophotometer
Pronunciation link: https://www.howtopronounce.com/spectrophotometer
IPA: /ˌspɛktroʊfəˈtɑːmɪtər/
Phonetic Spelling: spek-troh-fuh-tah-muh-ter

9. Deionized
Pronunciation link: https://www.merriam-webster.com/dictionary/deionized
IPA: /diˈaɪəˌnaɪzd/
Phonetic Spelling: dee-eye-uh-nyzd

10. SHP-2 (Src homology region 2 domain-containing phosphatase-2)
Pronunciation link: https://www.howtopronounce.com/shp-2
IPA: /ɛs-eɪtʃ-pi tuː/
Phonetic Spelling: ess-aitch-pee too

11. DTT (Dithiothreitol)
Pronunciation link: https://www.howtopronounce.com/dtt
IPA: /diː-tiː-tiː/
Phonetic Spelling: dee-tee-tee

12. Parafilm
Pronunciation link: https://www.howtopronounce.com/parafilm
IPA: /ˈpærəˌfɪlm/
Phonetic Spelling: pair-uh-film

13. Cytometer
Pronunciation link: https://www.howtopronounce.com/cytometer
IPA: /saɪˈtɑːmɪtər/
Phonetic Spelling: sigh-tah-muh-ter

14. Streptavidin
Pronunciation link: https://www.howtopronounce.com/streptavidin
IPA: /strɛpˈtævɪdɪn/
Phonetic Spelling: strep-ta-vih-din