FINAL SCRIPT: APPROVED FOR FILMING

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Title: Live-Cell Imaging of Endocytic Transport using Functionalized Nanobodies in Cultured Cells

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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, all done**

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

   It is only on different flowers in the same building

4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **YES**

Current Protocol Length
Number of Steps: 19
Number of Shots: 39

Introduction

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

INTRODUCTION:

NOTE: Authors have altered almost all statements to reflect the same content but in a slightly different phrasing. Edits made have not been mentioned

1.1. **Dominik P. Buser:** We are studying the endocytic and retrograde traffic of transmembrane proteins, as well as the machinery that confers their transport.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:3.7*

1.2. **Dominik P. Buser:** To investigate traffic from the cell surface, conventional antibodies are used but may promote crosslinking and lysosomal targeting.

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

CONCLUSION:

1.3. **Dominik P. Buser:** We have established a versatile nanobody-based toolkit to analyze transport of any transmembrane protein from the cell surface.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:2.10*

1.4. **Kai D. Schleicher:** We employ fluorescent nanobodies to label surface cargo proteins and visualize their endocytic trafficking by live-cell imaging.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:4.3*

1.5. **Kai D. Schleicher:** With our nanobody-based live-cell imaging approach, we seek to uncover sorting pathways driving surface-to-TGN cargo transport.

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.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Dominik P. Buser, Lecturer and Senior Scientist** : (authors will present their testimonial statements live)

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7. **Dominik P. Buser, Lecturer and Senior Scientist**: (authors will present their testimonial statements live)

Protocol

2. Purification of His-Tagged VHH-mCherry from Transformed *E. coli* Using Immobilized Metal Affinity Chromatography (IMAC)

Demonstrator: Dominik P. Buser and Tina Junne

2.1. To begin, obtain a pellet of *Escherichia coli* bacteria transformed with VHH-mCherry *(V-H-H-M-Cherry)* **[1].** Add 30 milliliters of ice-cold binding buffer containing 20 millimolar imidazole in PBS to the bacterial cell pellet **[2]**.

2.1.1. WIDE: Talent holding a labeled tube containing transformed bacterial pellet.

2.1.2. Talent adding 30 mL ice-cold binding buffer to the bacterial pellet.

2.2. With a pipette, resuspend the pellet thoroughly by pipetting up and down **[1]**. Transfer the resuspended mixture into a labeled 50-milliliter centrifuge tube **[2]**.

2.2.1. Talent pipetting the cell mixture repeatedly to resuspend thoroughly.

2.2.2. Talent transferring the suspension into a labeled 50 milliliter centrifuge tube.

2.3. Supplement the resuspended cells with 200 micrograms per milliliter lysozyme, 20 micrograms per milliliter DNase I, 1 millimolar magnesium chloride, and 1 millimolar phenylmethylsulfonyl fluoride **[1]**. After incubating the tube at room temperature for 10 minutes, place it on an end-over-end rotator and continue incubation at 4 degrees Celsius for 1 hour **[2]**.

2.3.1. Talent adding lysozyme, DNase I, magnesium chloride, and phenylmethylsulfonyl fluoride to the resuspended cells.

2.3.2. Talent placing the tube onto an end-over-end rotator set inside a cold room or refrigerated environment.

2.4. Next, insert a 6-millimeter solid probe tip of the sonicator directly into the cell suspension **[1]**. Mechanically disrupt the bacterial cells using three 1-minute sonication pulses at 40 percent amplitude and a duty cycle of 1 second on and 1 second off, allowing a 1-minute cooling interval between each pulse **[2]**.

2.4.1. Talent positioning the sonicator probe tip directly into the cell mixture.

2.4.2. Talent operating the sonicator under the specified settings, showing cooling intervals.

2.5. ~~Clarify the lysate by placing the tube in a centrifuge at 15,000 *g* at 4 degrees Celsius for 45 minutes to pellet the bacterial cell debris and intact bacteria **[1].**~~

2.5.1. ~~Talent placing the tube into the centrifuge and closing the lid to start the run.~~
NOTE: 2.5 deleted by authors

2.6. After centrifugation, keep the cleared lysate on ice **[1]** while preparing for immobilized metal affinity chromatography using pre-packed, single-use His-tag purification columns designed for gravity-flow operation **[2]**.

2.6.1. Talent placing the cleared lysate tube on ice.

2.6.2. Talent selecting gravity-flow His-tag purification columns from storage.

2.7. Secure nickel-nitrilotriacetic acid columns onto a metal stand or an appropriate column holder **[1]**. Then drain the storage buffer from the column completely **[2].**

2.7.1. Talent attaching the Ni-NTA columns onto a column rack or stand.

2.7.2. Talent draining the storage buffer from the purification column.

2.8. Equilibrate the column by adding 10 milliliters of binding buffer containing 20 millimolar imidazole in PBS ~~**[1].**~~ . Allow the buffer to pass through by gravity ~~and discard the flow-through as biowaste~~ **[1]**.

2.8.1. Talent pouring binding buffer into the column for equilibration.

2.8.2. ~~Talent discarding the flow-through into a biowaste container.~~

2.9. Gradually apply approximately 30 milliliters of the cleared bacterial lysate to the equilibrated column **[1]**. Allow it to pass through by gravity and discard the flow-through **[2]**. Then wash the column by applying two consecutive 10 milliliter volumes of binding buffer containing 20 millimolar imidazole in PBS **[3]**.

2.9.1. Talent pouring 30 mL cleared lysate into the column.

2.9.2. Talent discarding the lysate flow-through.

2.9.3. Talent performing washes using binding buffer.

2.10. Elute the bound nanobodies by applying 2 milliliters of elution buffer containing 500 millimolar imidazole in PBS into a 2-milliliter microcentrifuge tube **[1]**.

2.10.1. Talent eluting nanobodies into a 2-milliliter tube using the elution buffer.

2.11. Equilibrate a desalting column seated in a 50-milliliter tube adapter by rinsing five times with 5 milliliters of PBS **[1]**. Let the buffer fully enter the packed resin, then discard the flow-through **[2]**. After the final rinse, centrifuge the column at 1,000 *g* for 2 minutes **[3]**.

2.11.1. Talent rinsing the desalting column with 1x PBS.

2.11.2. Talent discarding the flow-through after the buffer enters the resin.

2.11.3. Talent placing the column into a centrifuge and spinning.

2.12. Now move the column with its adapter onto a new 50-milliliter collection tube

after discarding the flowthrough **[1]**. Load 2 milliliters of the eluted functionalized nanobody onto the pre-equilibrated desalting column **[2]**, then centrifuge again at 1,000 *g* for 2 minutes to collect the eluate before validating VHH-mCherry expression and purity **[3]**.

2.12.1. Talent placing the column over a new collection tube.

2.12.2. Talent loading 2 milliliters of eluted nanobody solution onto the column.

2.12.3. Talent centrifuging the column to collect the buffer-exchanged eluate.

3. Live-Cell Imaging and Quantitative Analysis of VHH-mCherry Endocytosis Using Time-Lapse Fluorescence Microscopy

Demonstrator: Dominik P. Buser and Kai D. Schleicher

3.1. Launch the software of the automated live-cell imaging system ~~pre-heat the microscope's incubation chamber to 37 degrees Celsius with 5 percent carbon dioxide~~ **[1]**. Transfer the ibidi microscopy chamber onto the microscope stage **[2].** ~~and configure the acquisition settings **[3].**~~

3.1.1. SCREEN: 69284_3.1.1.mp4 00:00-00:10

3.1.2. Talent positioning the ibidi chamber onto the microscope stage.

3.1.3. ~~SCREEN: Show configuration of acquisition settings on the imaging software.~~
NOTE: Shot deleted by authors

3.2. Set up two imaging channels using excitation and emission filters for EGFP and mCherry **[1-TXT]**. Choose a short exposure time and low illumination intensity to avoid photobleaching. Keep the settings constant for all experiments. Settings might differ between reporters **[2].**

3.2.1. SCREEN: 69284_3.2.1.mp4. 00:07-00:30
TXT: EGFP ex: 470/20 nm, em: 517/20 nm;
mCherry ex: 550/15 nm, em: 590/20 nm

3.2.2. SCREEN: 69284_3.2.2.mp4. 00:05-00:30

3.3. For each condition, store coordinates of 10 different fields of view containing 3 to 4 cells in focus in a point list **[1]**. Then capture a single snapshot of each position in the point list to serve as the reference image before adding VHH-mCherry **[2]**.

3.3.1. SCREEN: 69284_3.3.1.mp4 00:05-00:10, 00:26-00:30,00:50-01:10

3.3.2. SCREEN: 69284_3.3.2.mp4 00:00-00:12

3.4. To initiate endocytosis, gently add 350 microliters of pre-warmed VHH-mCherry solution

to each well while minimizing disturbance to the monolayer **[1]**. ~~Incubate at 37 degrees Celsius with 5 percent carbon dioxide~~ Then proceed with image acquisition **[2]**.

3.4.1. Talent gently adding VHH-mCherry solution into the ibidi wells.

3.4.2. ~~Talent placing the chamber back into the incubation chamber.~~

3.4.2. Talent sitting down on chair and proceeding with the image acquisition.

3.5. Perform a time-lapse acquisition for 100 frames at 30 second intervals while maintaining 37 degrees Celsius and 5 percent carbon dioxide **[1]**. ~~Order the channel acquisition sequence to image mCherry first, followed by EGFP to reduce photobleaching **[2]**.~~
NOTE: Shot deleted by authors

3.5.1. SCREEN: 69284_3.5.1.mp4 00:14-00:37

3.5.2. ~~SCREEN: Configure channel acquisition order in software.~~

3.6. ~~Increase throughput by acquiring all positions sequentially and repeating this sequence at each time point **[1]**.~~ **NOTE: Shot deleted by authors**

3.6.1. ~~SCREEN: Display sequential acquisition schedule across all time points~~.

3.7. To analyze endocytic uptake of VHH-mCherry, load the time-lapse image sets into the image analysis software **[1]**. Apply segmentation and tracking tools to quantify internalized fluorescence over time across different regions of interest **[2]**.

3.7.1. SCREEN: 69284_3.7.1.mp4. 00:03-00:20

3.7.2. SCREEN: 69284_3.7.2.mp4. 00:00-00:15

Results

4. Results

4.1. All functionalized nanobody variants were purified to high yield and purity **[1]**, with only VHH-mCherry exhibiting minor proteolytic degradation **[2]**. The degradation products of VHH-mCherry were confirmed as individual VHH and mCherry domains using epitope-specific immunoblot detection **[3]**.

4.1.1. LAB MEDIA: Figure 1B.

4.1.2. LAB MEDIA: Figure 1B. *Video editor: Highlight the bands in lane 5 (VHH-mCherry) of the gel*

4.1.3. LAB MEDIA: Figure 1C. *Video editor: Highlight the bands in lane 5 across the anti-HA, anti-T7, anti-His6, and SA-HRP blots*

4.2. In the absence of BirA *(Birr-A)*, VHH-mCherry was recovered at approximately 20 milligrams per preparation, and biotinylation was confirmed by streptavidin-agarose pulldown of nanobody-BSA *(B-S-A)* mixtures **[1]**.

4.2.1. LAB MEDIA: Figure 1D. *Video editor: Highlight lanes 9 and 10*

4.3. EGFP*(E-G-F-P)*-tagged fusion proteins expressed in HeLa *(Hee-lah)* cells showed subcellular localization patterns consistent with their endogenous counterparts **[1]**, including perinuclear localization of EGFP-CDMPR*(E-G-F-P-C-D-M-P-R)* and EGFP-CIMPR *(E-G-F-P-C-I-M-P-R)* **[2]**, exclusive perinuclear localization of EGFP-TGN46 *(E-G-F-P-T-G-N-Forty-Six)* **[3]**, and peripheral localization of TfR-EGFP *(T-F-R-E-G-F-P)* **[4]**.

4.3.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the green and red channels in the first row for EGFP-CDMPR and CDMPR (top row)*

4.3.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the green and red channels in the second row for EGFP-CIMPR and CIMPR, (2nd row)*

4.3.3. LAB MEDIA: Figure 2B. *Video editor: Highlight the green and red channels in the third row for EGFP-TGN46 and TGN46 (3rd row)*

4.3.4. LAB MEDIA: Figure 2B. *Video editor: Highlight the green and red channels in the fourth row for TfR-EGFP and TfR (bottom row)*

4.4. VHH-mCherry enabled live-cell visualization of endocytic transport of EGFP-CDMPR, showing increasing colocalization over 60 minutes **[1]**. Uptake of VHH-mCherry into EGFP-CDMPR-expressing cells reached a plateau after approximately 43 minutes, with a half-life of 9 minutes **[2]**. In TfR-EGFP-expressing cells, VHH-mCherry rapidly accumulated intracellularly, with a half-life of 4 minutes and saturation after 20 minutes **[3]**.

4.4.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the merged images at 0, 10, 20, 30,*

and 60 minutes, showing progressive yellow overlap

4.4.2. LAB MEDIA: Figure 3C. *Video editor: Highlight the curve showing saturation near the 40-minute mark*

4.4.3. LAB MEDIA: Figure 3D. *Video editor: Highlight the curve leveling off near the 20-minute mark*

Pronunciation Guide:

 nanobody
Pronunciation link: https://www.howtopronounce.com/nanobody
IPA: /ˈnænoʊˌbɒdi/
Phonetic Spelling: NAN-oh-BAH-dee

 VHH (variable heavy domain of heavy-chain antibodies)
Pronunciation link: No confirmed link found
IPA: /ˌviːˌeɪtʃˈeɪ/
Phonetic Spelling: vee-aych-ay

 mCherry
Pronunciation link: https://www.howtopronounce.com/mcherry howtopronounce.com
IPA: /məˈtʃɛri/
Phonetic Spelling: muh-CHER-ee

 phenylmethylsulfonyl fluoride
Pronunciation link: https://www.howtopronounce.com/phenylmethylsulfonyl-fluoride howtopronounce.com
IPA: /ˌfɛnɪlˌmɛθəlˈsʌlfoʊnaɪl ˈfluəraɪd/
Phonetic Spelling: FEN-il-meth-uhl-SUL-foh-nyl FLUOR-ide

 endocytic
Pronunciation link: https://www.howtopronounce.com/endocytic (if available)
IPA: /ˌɛn.doʊˈsɪtɪk/
Phonetic Spelling: en-doh-SIT-ik

 retrograde
Pronunciation link: https://dictionary.cambridge.org/us/pronunciation/english/retrograde ([Cambridge Dictionary]) Cambridge Dictionary
IPA: /ˈrɛtrəˌgreɪd/
Phonetic Spelling: RET-ruh-grade

 TGN46 (as in the protein “T-G-N forty-six”)
Pronunciation link: No confirmed link found
IPA: /ˌtiː-dʒiː-ˈɛn ˌfɔːrti-sɪks/
Phonetic Spelling: tee-gee-en forty-six

 IMAC (immobilized metal affinity chromatography)
Pronunciation link: No confirmed link found
IPA: /ˌaɪ ˌɛm-ˈeɪ ˌsiː/
Phonetic Spelling: eye-em-AY-cee

 segmentation
Pronunciation link: https://dictionary.cambridge.org/us/pronunciation/english/segmentation Cambridge Dictionary
IPA: /ˌsɛg.mənˈteɪʃən/
Phonetic Spelling: seg-mən-TAY-shun

 colocalization
Pronunciation link: https://dictionary.cambridge.org/us/pronunciation/english/colocalization (if available)

IPA: /ˌkoʊˌloʊkələˈzeɪʃən/
Phonetic Spelling: coh-loh-coh-luh-ZAY-shun