FINAL SCRIPT: APPROVED FOR FILMING

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# Title: Live-Cell Förster Resonance Energy Transfer Imaging of Metabolically Regulated Akt Activation Dynamics in HepG2 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**

   If **Yes**, how far apart are the locations? **Approximately 30 kilometers apart.**

**Current Protocol Length**
Number of Steps: 25
Number of Shots: 55 (22 SC)

# Introduction

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

1.1. **Guanyu Wang:** Cellular heterogeneity is an obstacle to the accurate description of intricacies of cellular dynamics, which are essentially averaged out. That is why we are here to image single cells.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.1*

What are the most recent developments in your field of research?

1.2. **Gang Ma:** Recent developments in genetically encoded FRET biosensors have improved specificity and revolutionized real-time tracking of Akt within cells, which is instrumental in understanding heterogeneity and developing personalized therapies.

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

What technologies are currently used to advance research in your field?

1.3. **Javed Akhtar:** Cancer and diabetes are interlinked through complex metabolic pathways. To decode the biocomplexity of the insulin signaling pathway in cancer, we used cutting-edge live-cell FRET imaging at single-cell resolution.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2.1*

What are the current experimental challenges?

1.4. **Muhammad Imran:** We found that optimizing confocal microscope hardware and software configurations, along with fine-tuning laser power and adjusting detector gain, is critical to minimize image noise and spectral crosstalk in FRET signals.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.2.1*

What research gap are you addressing with your protocol?

1.5. **Jiahe Wang:** Traditional bulk methods, like Western blotting, lack spatiotemporal detail and mask cellular heterogeneity. Our protocol fills this gap by using live-cell FRET imaging to capture cellular responses with high precision and accuracy.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.6.1*

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

**Testimonial Questions (OPTIONAL):**

***Videographer: Please ensure that all testimonial shots are captured in a wide-angle format, while also maintaining sufficient headspace, given that the final videos will be rendered in a 1:1 aspect ratio.***

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

1.6. **Javed Akhtar:** Publishing with JoVE will enhance the visibility of our FRET protocol by making complex imaging techniques easier to follow and reproduce. This multimedia approach attracts a wider audience, improves reproducibility, encourages interdisciplinary collaboration, and increases the potential for real-world impact and citations.

1.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.6.1*

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE?

1.7. **Javed Akhtar:** We expect that publishing our FRET protocol with JoVE will increase collaboration requests from researchers focused on metabolic diseases. The visual format streamlines lab training, significantly reduces setup time for new researchers, and will enhance the protocol's visibility, potentially increasing the citations.

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

# Protocol

## 2. Coating Imaging Dishes with Poly-L-Lysine

**Demonstrator:** Jiahe Wang

2.1. To begin, add 1 milliliter of a 0.1 milligram per milliliter Poly-L-Lysine solution to an imaging dish **[1]** and rock the dish to cover the entire surface **[2]**. After overnight incubation, aspirate the excess Poly-L-Lysine solution from the dish **[3]**.

2.1.1. WIDE: Talent pipetting 1 milliliter of Poly-L-Lysine solution onto an imaging dish.

2.1.2. Talent gently rocking the dish by hand to coat the surface evenly.

2.1.3. Talent using a pipette to remove excess Poly-L-Lysine solution.

2.2. Gently rinse the surface with PBS three times, resting for 5 minutes each, to completely remove the unbound Poly-L-Lysine **[1-TXT]**. Aspirate the PBS **[2]** and air dry the coated imaging dish at 37 degrees Celsius for at least 3 hours **[3]**. **NOTE**: VO added for the extra shot

2.2.1. Talent pipetting phosphate-buffered saline onto the dish. **TXT: Avoid scraping or damaging the glass bottom**

Added shot: Talent aspirating the PBS.

2.2.2. Talent placing the dish inside an incubator set at 37 degrees Celsius.

2.3. Next, seed the HepG2 *(hep-G-2)* cells in the pre-coated imaging dish 24 to 48 hours prior to transfection to ensure they reach 70 to 90% confluence **[1]**. Thaw and vortex the transfection reagent along with the plasmid encoding the Förster Resonance Energy Transfer biosensor **[2]**. Perform a short spin to collect all the mixture at the bottom of the tube **[3-TXT]. NOTE**: VO added for the extra shot

2.3.1. Talent pipetting HepG2 cells into pre-coated imaging dishes.

2.3.2. Talent vortexing the thawed plasmid.

Added shot: Talent performing a short spin to collect the mixture. **TXT: Perform a short spin before use.**

2.4. Mix 8 micrograms of the plasmid with the reaction buffer to a final volume of 100 microliters using a vortex for 5 seconds at high speed **[1]**. Perform a short spin to collect the mixture at the bottom of the tube **[2]** Add 2.4 microliters of the transfection polymer to the tube containing the diluted plasmid DNA and mix well **[3]**. **NOTE**: VO added for the extra shot

2.4.1. Talent vortexing the mixture solution.

Added shot: Talent performing a short spin to collect the mixture at the bottom of the tube.

2.4.2. Talent pipetting the transfection polymer into the reaction tube.

2.5. Incubate the biosensor and polymer mixture at 37 degrees Celsius for 15 minutes to allow nanoparticle complexes to form **[1]**.
  2.5.1. Talent placing the reaction tube inside a 37-degree Celsius incubator.

2.6. Spin down the tube for 5 seconds at 5,000 *g* to collect the contents at the bottom **[1]**. Add the entire 100 microliters of nanoparticle complex solution dropwise to the cell culture medium **[2]** and gently rock the plate back and forth to mix **[3]**. Incubate the plate at 37 degrees Celsius for 4 hours to overnight **[4]**.
  2.6.1. Talent placing the sample in the centrifuge.
  2.6.2. Talent pipetting 100 microliters of nanoparticle complex solution dropwise into the culture medium.
  2.6.3. Talent gently rocking the dish by hand to coat the surface evenly.
  2.6.4. Talent placing the plate inside an incubator.

2.7. After removing the nanoparticle complexes from the cells, replace the solution with 2 milliliters of fresh complete growth medium **[1]**. Return the plate to the 37-degree Celsius incubator until the time of analysis **[2-TXT]**. Analyze the cells under fluorescence microscopy **[3]**.
  2.7.1. Talent aspirating transfection reagent from the plate and adding fresh media.
  2.7.2. Talent placing the plate back into the incubator. **TXT: Peak expression typically reaches 48 h post-transfection**
  2.7.3. Talent working at the fluorescence microscope unit.

## 3. Starvation and Förster Resonance Energy Transfer (FRET) Live-Cell Imaging of HepG2 Cells

**Demonstrator:** Javed Akhtar

3.1. Remove the culture medium from the imaging dish **[1]** and rinse the dish twice with 1X PBS, allowing each rinse to last for five minutes **[2]**. Add two milliliters of starvation medium to each dish around the edge of the dish **[3]**.
  3.1.1. Talent removing culture medium from the imaging dish.
  3.1.2. Talent pipetting 1X phosphate-buffered saline into the dish and gently shaking the plate.
  3.1.3. Talent adding starvation medium into the dish.

3.2. Incubate the cells at 37 degrees Celsius for four hours **[1]**.
  3.2.1. Talent placing the dish inside the incubator.

3.3. Next, turn on the computer **[1]**. Then, power on the confocal microscope main unit and accessories sequentially **[2]**. After that, activate the laser system by turning the key to the ON position and flipping the laser launch toggle switches **[3]**. Then, rotate the laser lock switches clockwise to the ON position for both the 457-nanometer and 514-nanometer laser lines **[4]**. Finally, turn on the system controller power **[5]**. **NOTE**: VO and shots were changed for the entire step

3.3.1. Close-up shot of talent turning on the computer.
3.3.2. Talent pressing the main power buttons on the microscope and accessories.
3.3.3. Talent turning the key to the ON position and flipping the laser launch toggle switches.
3.3.4. Talent rotating both laser lock switches clockwise to the ON position.
3.3.5. Talent powering on the system controller.

3.4. Launch the NIS-Elements AR software from the desktop **[1]**.
3.4.1. SCREEN: 68075_screenshot_1.mp4. 00:05-00:18

3.5. Click on **A1 for Acquisition** to initiate the imaging setup **[1]** and select the appropriate optical configurations based on the experimental requirements **[2-TXT]**.
3.5.1. SCREEN: 68075_screenshot_2.mp4 00:00-00:12.
3.5.2. SCREEN: 68075_screenshot_2.mp4 01:00-01:10. **TXT: Adjust the laser power and detector sensitivity as needed**

3.6. Install the microscope stage top incubator and secure it with screws **[1]**. Fill the internal water bath with sterile double-distilled water **[2]**. Then, install the top heater and turn on the power supply for the stage heater, bath heater, and lens heater **[3]**.
3.6.1. Talent securing the microscope stage incubator with screws.
3.6.2. Talent filling the internal water bath with sterile double-distilled water.
3.6.3. Talent turning on the power supply for the heaters.

3.7. Wipe the 40X oil immersion lens with lens paper moistened with 95 percent ethanol **[1]** and place a small droplet of immersion oil on the objective lens **[2]**. Then, position the imaging dish containing the HepG2 cells onto the microscope stage and secure it with the holder **[3]**.
3.7.1. Talent cleaning the objective lens with ethanol-moistened lens paper.
3.7.2. Close-up shot of immersion oil being applied to the lens.
3.7.3. Talent placing the imaging dish on the stage and securing it.

3.8. Close the chamber and incubate the cells within the live-cell imaging chamber for one to two hours to allow for equilibration **[1]**.
3.8.1. Talent closing the imaging chamber.

~~3.9. During time-lapse imaging, pause at specific intervals to gently remove the medium from the dish **[1]** and add one milliliter of freshly prepared medium containing the given insulin concentration **[2]**.~~ **NOTE**: Not filmed

~~Talent pipetting out the medium from the imaging dish.~~

~~Talent adding freshly prepared medium to the dish~~.

3.10. Launch the **ND Acquisition** window from the **File** menu of the NIS *(N-I-S)*-Elements AR **[1]**.

3.10.1. SCREEN: 68075_screenshot_3.mp4 00:00-00:05.

3.11. Select the **Time** tab to set the interval, duration, and loops for time-lapse imaging **[1]**. Click on the **XY** tab and press the **+ Add** *(add)* button to include HepG2 cells for imaging under focus **[2]**. Then, check the Z *(Zee)* box to set the Z position **[3]**.

3.11.1. SCREEN: 68075_screenshot_4.mp4 00:00-00:10.

3.11.2. SCREEN: 68075_screenshot_4.mp4 00:10-00:20.

3.11.3. SCREEN: 68075_screenshot_4.mp4 00:20-00:27.

~~3.12. To add multiple cells, locate suitable cells, scan, fine-tune the focus, and lock the Point Spread Function **[1]**. Scan multiple locations within the dish to identify various target cells **[2]**.~~

~~3.12.1. SCREEN: Scanning for suitable cells and adjusting focus.~~

3.12.2. ~~SCREEN: Selecting multiple target cells from different locations~~.

3.13. Click **Browse** to select a destination folder **[1]**. Enter the experiment name in the **File Name** box **[2]**. Click **Run Now** to initiate acquisition **[3]** and start the time-lapse imaging process **[4]**.

3.13.1. SCREEN: 68075_screenshot_5.mp4 00:00-00:08.

3.13.2. SCREEN: 68075_screenshot_5.mp4 00:08-00:32.

3.13.3. SCREEN: 68075_screenshot_5.mp4 00:32-00:42.

3.13.4. SCREEN: 68075_screenshot_5.mp4 00:42-01:00.

3.14. Record baseline measurements for up to 30 minutes without insulin-supplemented media **[1]**.

3.14.1. SCREEN: 68075_screenshot_6.mp4 00:00-00:20.

3.15. Pause imaging at regular intervals **[1]**. Gently remove the medium from the glass-bottom dish and add one milliliter of medium supplemented with the appropriate insulin concentration **[2]**.

3.15.1. SCREEN: 68075_screenshot_6.mp4 00:20-00:40.

3.15.2. Talent replacing the medium with insulin-supplemented medium. **NOTE**: This may have been slated as 3.15.2 or 3.17.2. If the footage is not found, let me know (poornima.g@jove.com) and we can move the VO as on-screen text

3.16. Resume the image acquisition **[1]** and click **Finish** at the end of the experiment to close **[2]**. Finally, back up and securely store acquired images for analysis **[3]**.
3.16.1. SCREEN: 68075_screenshot_7.mp4 00:00-00:27.
3.16.2. SCREEN: 68075_screenshot_7.mp4 00:27-00:40.
3.16.3. SCREEN: 68075_screenshot_7.mp4 00:40-00:50.

# Results

**4. Results**

4.1. A dose-dependent increase in Akt *(A-K-T)* phosphorylation was observed upon insulin stimulation, with a sharp increase occurring at 300 picomolar insulin **[1]**. Beyond this concentration, phosphorylation levels plateaued and did not come down even after insulin was decreased **[2]**.

4.1.1. LAB MEDIA: Figure 10A. *Video editor: Highlight the sharp rise in line graph corresponding to "300" mark on the X-axis*.

4.1.2. LAB MEDIA: Figure 10A. *Video editor: Highlight the line graph corresponding to "400 ↑ " on the X-axis till the end*.

4.2. The normalized FRET *(fret)* ratio showed a similar trend, with an increasing signal upon insulin stimulation **[1]** and failure to return to baseline upon insulin withdrawal **[2]**.

4.2.1. LAB MEDIA: Figure 10B. *Video editor: Mark the orange ascent line*.

4.2.2. LAB MEDIA: Figure 10B. *Video editor: Mark the blue descent line*.

4.3. Upon sequential insulin stimulation, a progressive increase in the normalized FRET ratio was observed, supporting the dose-dependent Akt activation response **[1]**.

4.3.1. LAB MEDIA: Figure 10C.

Pronunciation guide

## 1. Poly-L-Lysine

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/polylysine
- **IPA**: /ˌpɑː.liˈlaɪ.siːn/
- **Phonetic Spelling**: PAH-lee-LY-seen(merriam-webster.com)

## 2. Phosphate-Buffered Saline (PBS)

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/phosphate
- **IPA**: /ˈfɑːs.feɪt ˈbʌf.ərd ˈseɪ.liːn/
- **Phonetic Spelling**: FOS-fate BUFF-erd SAY-leen(merriam-webster.com)

### 3. HepG2

- **Pronunciation link**: No confirmed link found
- **IPA**: /ˌhɛp.dʒiːˈtuː/
- **Phonetic Spelling**: HEP-jee-TOO

### 4. Transfection

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/transfection
- **IPA**: /trænsˈfɛk.ʃən/
- **Phonetic Spelling**: trans-FEK-shun(merriam-webster.com)

### 5. Plasmid

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/plasmid
- **IPA**: /ˈplæz.mɪd/
- **Phonetic Spelling**: PLAZ-mid(merriam-webster.com)

### 6. Förster Resonance Energy Transfer (FRET)

- **Pronunciation link**: No confirmed link found
- **IPA**: /ˈfɜːr.stər ˈrɛz.ə.nəns ˈɛn.ər.dʒi ˈtræns.fər/
- **Phonetic Spelling**: FUR-stur REZ-uh-nuhns EN-er-jee TRANS-fur

### 7. Biosensor

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/biosensor
- **IPA**: /ˈbaɪ.oʊˌsɛn.sər/
- **Phonetic Spelling**: BY-oh-sen-sor(merriam-webster.com)

### 8. Nanoparticle

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/nanoparticle
- **IPA**: /ˈnæn.oʊˌpɑːr.tɪ.kəl/
- **Phonetic Spelling**: NAN-oh-par-ti-kl

### 9. Akt

- **Pronunciation link**: No confirmed link found
- **IPA**: /ˈeɪ.kay.ti/
- **Phonetic Spelling**: AY-kay-tee