

Submission ID #: 70151

Scriptwriter Name: Deepthi Das

Project Page Link: <https://review.jove.com/account/file-uploader?src=21396103>

Title: Super-Resolution Imaging and Shared Management: A Protocol for Confocal Microscopy with Multiplex Detection

Authors and Affiliations:

Wei Yin¹, Guifeng Xiao¹, Sanhua Fang¹, Qianbing Zhao¹, Qiong Huang¹, Wei Wei², Xianzhu Zhang³

¹Core Facilities, Zhejiang University School of Medicine

²Department of Orthopedics, the Fourth Affiliated Hospital, Zhejiang University School of Medicine

³Department of Sports Medicine of the First Affiliated Hospital, Zhejiang University School of Medicine

Corresponding Authors:

Wei Wei (zjewwei@zju.edu.cn)

Xianzhu Zhang (zhangxianzhu@zju.edu.cn)

Email Addresses for All Authors:

yinwei5651@zju.edu.cn

xiaogf@zju.edu.cn

fshfbzxhq@zju.edu.cn

zhaoqb@zju.edu.cn

huangqiong@zju.edu.cn

zjewwei@zju.edu.cn

zhangxianzhu@zju.edu.cn

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? **No**
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. **No**

Current Protocol Length

Number of Steps: 22

Number of Shots: 37 (32 SC)

Introduction

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

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Wei Yin:** Our research focuses on developing accessible super-resolution imaging methods to visualize dynamic subcellular structures, overcoming the diffraction limit of conventional microscopy.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Wei Yin:** Advanced super-resolution methods face phototoxicity, complex operation, and specialized preparation; this protocol reduces these barriers through confocal-based multiplex detection
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Wei Yin:** This protocol allows researchers to study organelle morphology, cytoskeleton dynamics, and molecular co-localization in both fixed and living cells at approximately 120 nanometer resolution.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Wei Yin:** The most critical consideration of this protocol is meticulous sample preparation using high-quality cover glass and optimized staining to ensure signal integrity for super-resolution processing.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Wei Yin:** Following this procedure, researchers can perform quantitative image analysis, such as measuring signal-to-noise ratio or co-localization, using dedicated software like ImageJ.
 - 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. Microscope System Startup, Initialization, and Basic Alignment

Demonstrator: Wei Yin

- 2.1. To begin, power on the microscope main unit, the laser launch module, the LED (L-E-D) light source, and the control computer [1-TXT].
 - 2.1.1. WIDE: Talent turning on microscope main unit, laser launch module, the LED light source, and control computer. **TXT: Let the lasers warm up and stabilize for 15-20 min**
- 2.2. Launch the microscope operating software [1]. From the main interface, select **GFP**, then choose turn the **Reflected Light** off [2].
 - 2.2.1. SCREEN: 70151_2.2.1_screenshot_1.mp4 00:04-00:12 .
 - 2.2.2. SCREEN: [70151 2.2.2 screenshot 2.mp4](#) 00:00-00:17
- 2.3. , Navigate to the **Acquisition** panel. From the objective lens dropdown menu, select the appropriate objective lens [1].
 - 2.3.1. SCREEN: [70151 2.3.1 screenshot 3.mp4](#) 00:00-00:07.
- 2.4. Apply immersion oil on the objective's front lens without introducing bubbles [1].
 - 2.4.1. Talent applying a drop of immersion oil on the lens.
AUTHOR'S NOTE: Please move 2.4 before 3.1
- 2.5. For live-cell imaging, forty-five to 60 minutes before imaging, activate the environmental chamber and the active focus stabilization system [1-TXT]. After the system is stabilized, verify the stability using the chamber sensors [2].
 - 2.5.1. Talent activating the environmental chamber and the active focus stabilization system. **TXT: Chamber settings – 37 °C, 5% CO₂**
 - 2.5.2. SCREEN: 70151_2.5.1_screenshot_4.mp4 00:16-00:28

3. Multicolor Fluorescence Imaging

- 3.1. Place the prepared sample on the microscope stage [1]. Raise it carefully until the coverslip contacts the immersion oil [2].
 - 3.1.1. Talent placing the prepared sample on the microscope stage.
 - 3.1.2. Talent raising the microscope stage until the coverslip contacts the immersion oil.
- 3.2. Next, open the acquisition software, go to the **Acquisition** tab, and select **Create a new experiment** [1].

- 3.2.1. SCREEN: 70151_3.2.1_screenshot_6.mp4 00:00-00:16 .
- 3.3. Now, add imaging tracks for the fluorophores used [1]. For each track, set the confocal pinhole diameter to **1 Airy Unit** [2].
- 3.3.1. SCREEN: [70151 3.3.1 screenshot 5.mp4](#) 00:07-00:12 .
- 3.3.2. SCREEN: [70151 3.3.2 screenshot 6.mp4](#) 00:00-end .
- 3.4. To optimize the laser power, select the first channel and switch to **Live** scanning mode [1]. Set the initial laser power to a low value [2]. Then, set **Detector Gain** to a moderate level within its linear range [3]. After that, increase the laser power gradually until the target structures are clearly visible above the background in the live image [4-TXT].
- 3.4.1. SCREEN: [70151 3.4.-and-3.5 screenshot 7.mp4](#). 00:02-00:03, 00:17-00:20
- 3.4.2. SCREEN: 70151_3.4.-and-3.5_screenshot_7.mp4. 00:04-00:07
- 3.4.3. SCREEN: 70151_3.4.-and-3.5_screenshot_7.mp4. 00:09-00:16
- 3.4.4. SCREEN: 70151_3.4.-and-3.5_screenshot_7.mp4. 00:24-00:37
TXT: Avoid gains above 850 V to prevent excessive noise
- ~~3.5. Then, open the **Histogram** display and reduce laser power, if necessary, to ensure there is no pixel saturation [1]. Adjust the **Detector Gain** to achieve a bright, low noise image, avoiding gains above 850 Volts to prevent excessive noise [2].~~
- ~~3.5.1. SCREEN: Opening the Histogram display and reducing laser power if necessary to prevent the histogram from piling up at the right edge.
Video editor: Put arrow or encircle the right edge of the histogram.~~
- ~~3.5.2. SCREEN: The Detector Gain is being adjusted to achieve a bright, low noise image.~~
- 3.6. After optimizing laser power and detector gain for all channels, **Set the pixel size** such that it is less than or equal to the resolution divided by 2.3. Use **using the software's Nyquist** sampling button to calculate automatically [1-TXT].
- 3.6.1. SCREEN: [70151 3.6-7-8-9 screenshot 8.mp4](#). 00:06-00:08 **TXT: Pixel size ≤108 nm for ~250 nm lateral resolution**
- 3.7. Next, set the pixel time. For higher signal to noise ratio, use a dwell time of one to two microseconds. For faster imaging, use half to one microsecond [1].
- 3.7.1. SCREEN: [70151 3.6-7-8-9 screenshot 8.mp4](#). 00:08-00:09

3.8. Now, select the **Sequential Scan** mode between tracks to minimize crosstalk [1].

3.8.1 SCREEN: [70151 3.6-7-8-9 screenshot 8.mp4](#). 00:09-00:15

3.9. Click **SNAP** to acquire a single image [1]. Save the image with relevant metadata [2].

3.9.1. SCREEN: [70151 3.6-7-8-9 screenshot 8.mp4](#). 00:28-00:42

3.9.2. SCREEN: 70151_3.9.2_screenshot_10.mp4 00:02-00:13.

4. Z-Stack Acquisition for Three-Dimensional Reconstruction

4.1. For consistent settings across optical sections, confirm that all imaging parameters are configured on a single, optimal focal plane before defining the Z-stack [1].

4.1.1. SCREEN: [70151 4.1.1 screenshot 10.mp4](#) 00:03-00:15.

4.2. Open the **Acquisition** tab and select the **Z-stack** mode [1].

4.2.1. SCREEN: [70151 4.2&4.3&4.4&4.5 screenshot 10.mp4](#) 00:00-00:05.

4.3. Define the volume of interest by using a fast-speed live scan to focus first on the topmost structure of interest [1]. Then, click **Set First** or **Top** [2]. Carefully adjust focus down to the bottommost structure of interest and click **Set Last** or **Bottom** [3]. The software will then display the total depth [4].

4.3.1. SCREEN: [70151 4.2&4.3&4.4&4.5 screenshot 10.mp4](#). 00:06-00:10

4.3.2. SCREEN: [70151 4.2&4.3&4.4&4.5 screenshot 10.mp4](#). 00:11-00:18

4.3.3. SCREEN: [70151 4.2&4.3&4.4&4.5 screenshot 10.mp4](#). 00:19-00:28

4.3.4. SCREEN: [70151 4.2&4.3&4.4&4.5 screenshot 10.mp4](#). 00:29-00:34

4.4. The Z-step size is set to meet the Nyquist sampling criterion. Use the **Optimal** button to calculate it automatically [1-TXT].

4.4.1. SCREEN: [70151 4.2&4.3&4.4&4.5 screenshot 10.mp4](#). 00:35-00:40

TXT: 63×/1.4 NA oil-immersion objective lens

4.5. After configuring the imaging parameters for each channel, increase laser power or gain slightly for weak signals compared to a single two-dimensional image [1].

4.5.1. SCREEN: [70151 4.2&4.3&4.4&4.5 screenshot 10.mp4](#). 00:41-end

4.6. Start the acquisition to collect the entire image stack. [1].

4.6.1. SCREEN: [70151 4.6&4.7 screenshot 13.mp4](#). 00:04-00:10

4.7. For visualization, use the microscope's native **Processing** module [1]. Generate maximum intensity projections to view all in-focus signals in a single two-dimensional

image [2]. Then, perform volume rendering using the software's built-in algorithms to generate a three-dimensional representation [3]. Adjust the brightness, opacity, and lighting as needed [4]. Subsequently, generate orthogonal XZ (X-Y) and YZ (Y-Z) cross-sections to inspect axial extent and registration [5].

- | | |
|--|--------------------------|
| 4.7.1. SCREEN: 70151 4.6&4.7 screenshot 13.mp4 . | 03:30-03:35 |
| 4.7.2. SCREEN: 70151 4.7.2 screenshot 14.mp4 | 00:03-00:15. |
| 4.7.3. SCREEN: 70151 4.7.3 screenshot 15.mp4 | 00:00-00:10 |
| 4.7.4. SCREEN: 70151 4.7.3 screenshot 15.mp4 | 00:11-00:20, 00:21-00:43 |
| 4.7.5. SCREEN: 70151 4.7.3 screenshot 15.mp4 | 00:51-01:08 |

Results

5. Results

- 5.1. Multicolor fluorescence imaging of fixed COS7 (*Cos-7*) cells [1] showed that the nuclei, mitochondria, and microtubules were clearly labeled with minimal overlap, confirming effective sequential scanning and optimized detection [2].

5.1.1. LAB MEDIA: Figure 1

5.1.2. LAB MEDIA: Figure 1 *Video Editor: Please put label next to the image as follows: Nuclei – Blue (DAPI); Mitochondria - Green (Alexa Fluor 488); Microtubules - Red (Alexa Fluor 568)*

- 5.2. The maximum intensity projection integrates signals across all optical sections, highlighting the spatial distribution of fluorescently labeled structures [1]. A three-dimensional reconstruction provides a depth aware view of the sample architecture [2].

5.2.1. LAB MEDIA: Figure 3B

5.2.2. LAB MEDIA: Figure 3C

- 5.3. Time-lapse imaging of live Hep3B (*hep-3-B*) cells with EGFP (*E-G-F-P*) -tagged mitochondria is compared across modes [1].

5.3.1. LAB MEDIA: Figure 4A, 4B, 4C *Video Editor: Please put label next to the image as follows: Mitochondria - Green (Alexa Fluor 488)*

- 5.4. Confocal frames across time points show clear structures, but gradual signal loss [1]. Standard super-resolution or SR (*S-R*) frames show higher resolution but increased photobleaching over time [2]. Fast super-resolution or SR-4Y (*S-R-4-Y*) images preserve stable fluorescence, even at later time points [3].

5.4.1. LAB MEDIA: Figure 4A

Video Editor: Please emphasize images labeled as A: Confocal t=1, A: Confocal t=50, A: Confocal t=100 and put label on top of the images as follows: Mitochondria - Green (Alexa Fluor 488)

5.4.2. LAB MEDIA: Figure 4B

Video Editor: Please emphasize images labeled as B: SR t=1, B: SR t=50, B: SR t=100 and put label on top of the images as follows: Mitochondria - Green (Alexa Fluor 488)

5.4.3. LAB MEDIA: Figure 4C

Video Editor: Please emphasize images labeled as C: SR-4Y t=1, C: SR-4Y t=50, C: SR-4Y t=100 and put label on top of the images as follows: Mitochondria - Green (Alexa Fluor 488)

- 5.5. The graph of mean EGFP fluorescence intensity over 100 time points demonstrates that the SR-4Y mode maintained the most stable signal [1].
 - 5.5.1. LAB MEDIA: Figure 4D *Video Editor: Please highlight the graph labeled SR-4Y*
- 5.6. Imaging performance in fixed COS-7 cells was compared across modes and super-resolution modes provided clearer structural detail than confocal [1].
 - 5.6.1. LAB MEDIA: Figure 6 *Video Editor: Please show the full image for 2 seconds and emphasize the difference in resolution between the figures 6B and 6F.*
- 5.7. Measuring the point spread function using 100 nanometer fluorescent beads showed that Airyscan SR with subsequent joint deconvolution improved both lateral and axial resolution compared to Airyscan SR alone [1].
 - 5.7.1. LAB MEDIA: Figure 8 *Video editor: Please show the full figure for 2 seconds and then emphasize the tables highlighted with orange rectangles.*

Pronunciation Guide:

1. Confocal
Pronunciation link: <https://www.merriam-webster.com/dictionary/confocal>
IPA: /kən'fəʊkəl/
Phonetic Spelling: kon·foh·kuhl
2. Microscopy
Pronunciation link: <https://www.merriam-webster.com/dictionary/microscopy>
IPA: /maɪ'krɑːskəpi/
Phonetic Spelling: my·kros·kuh·pee
3. Subcellular
Pronunciation link: <https://www.merriam-webster.com/dictionary/subcellular>
IPA: /ˌsʌb'sɛljələr/
Phonetic Spelling: sub·sel·yuh·ler
4. Cytoskeleton
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytoskeleton>
IPA: /ˌsaɪtəʊ'skɛlətən/
Phonetic Spelling: sy·toh·skel·uh·tuhn
5. Organelle
Pronunciation link: <https://www.merriam-webster.com/dictionary/organelle>
IPA: /ˌɔːrgə'nɛl/
Phonetic Spelling: or·guh·nel
6. Fluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /ˌflʊr'ɛsəns/
Phonetic Spelling: floor·es·uhns
7. Diffraction
Pronunciation link: <https://www.merriam-webster.com/dictionary/diffraction>
IPA: /dɪ'frækʃən/
Phonetic Spelling: dih·frak·shuhn
8. Mitochondria
Pronunciation link: <https://www.merriam-webster.com/dictionary/mitochondria>
IPA: /ˌmɪtə'kɑːndrɪə/
Phonetic Spelling: my·tuh·kon·dree·uh
9. Microtubules
Pronunciation link: <https://www.merriam-webster.com/dictionary/microtubule>
IPA: /ˌmaɪkrəʊ'tuːbjʊːlz/
Phonetic Spelling: my·kroh·toob·yoolz

10. Coverslip

Pronunciation link: <https://www.merriam-webster.com/dictionary/coverslip>

IPA: /'kʌvərˌslɪp/

Phonetic Spelling: kuv·er·slip

11. Phototoxicity

Pronunciation link: <https://www.merriam-webster.com/dictionary/phototoxicity>

IPA: /ˌfoʊtəʊˈtɒksɪsəti/

Phonetic Spelling: foh·toh·tok·sis·uh·tee

12. Multiplex

Pronunciation link: <https://www.merriam-webster.com/dictionary/multiplex>

IPA: /'mʌltəˌpleks/

Phonetic Spelling: mul·tuh·pleks