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**Title: A Streamlined Protocol for Single-Molecule Localization
Microscopy in *Arabidopsis* Nuclei**

Authors and Affiliations:

AUTHORS AND AFFILIATIONS:

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Alpes, EMBL

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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**

If **Yes**, we will need you to record using screen recording software.

We recommend using the screen capture program OBS. JoVE's tutorial for using OBS Studio is provided at this link: <https://review.jove.com/v/5848/screen-capture-instructions-for-authors?status=a7854k>

As these files are necessary for finalizing your script, **please upload all screen-captured video files to your project page as soon as possible.**

Videographer: Please record the computer screen for the shots labeled as SCREEN as back-up

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

If **Yes**, how far apart are the locations? **800m**

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: 23

Number of Shots: 53 (9 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. **Salim El-Khoury:** My research focuses on epigenome editing and the visualization of chromatin organization in *Arabidopsis* nuclei using super-resolution 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. **Salim El Khoury:** Existing methods are not adapted to STORM imaging; this protocol improves nuclei purity, labeling consistency, sample mounting and imaging stability.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research contexts or systems in which this protocol can be applied~~

- 1.3. **Salim El Khoury:** It applies to *Arabidopsis thaliana* and other plant systems for studying chromatin organization, epigenetics, and nuclear architecture at high resolution.
 - 1.3.1. 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. **Salim El Khoury:** This protocol enables nanoscale visualization of chromatin structure, histone modifications, and nuclear organization in individual plant nuclei.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

NOTE: This is not filmed

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

- 1.4. **Salim El Khoury:** Maintaining nuclear integrity while minimizing debris and background fluorescence is critical for achieving high-quality single-molecule imaging.

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

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Salim El Khoury:** Future studies can link nuclear architecture to gene regulation under developmental or environmental conditions.
 - 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

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

2. Fixation of *Arabidopsis* Seedlings

Demonstrator: Salim El-Khoury

- 2.1. To begin, place a 6-well culture plate on ice under the fume hood [1]. Mix the fixation solution containing 4% volume by volume methanol stabilized formaldehyde, 0.1% Triton X-100, and PBS [2]. Add approximately 4 milliliters of ice-cold fixation solution to each well under the fume hood [3].
 - 2.1.1. WIDE: Talent placing a 6-well culture plate on ice under the fume hood.
 - 2.1.2. Talent inverting the container with the fixation solution under the fume hood.
 - 2.1.3. Talent adding fixation solution to each well of the 6-well culture plate under the fume hood.
- 2.2. Using clean forceps, remove the *Arabidopsis* seedlings that are between 8 and 15 days after germination from solid half-strength Murashige and Skoog or MS medium [1]. Immediately submerge the seedlings in the fixation solution [2] and place approximately 20 seedlings per well [3]. Use 3 wells to obtain sufficient material per sample [4].
 - 2.2.1. Talent removing seedlings from the solid medium with forceps.
 - 2.2.2. Talent submerging the seedlings in the fixation solution.
 - 2.2.3. Talent placing approximately 20 seedlings into 1 well.
 - 2.2.4. Shot of seedlings added into 3 wells.
- 2.3. Place the plate in the vacuum chamber filled with ice and close it [1]. Apply a vacuum cycling between minus 0.6 bar and minus 0.8 bar for 15 minutes to remove trapped air and allow deep penetration of the fixative into the plant tissues [2]. Then, slowly release the vacuum [3]. Mix the sample and apply the vacuum for a further 15 minutes [4].
 - 2.3.1. Talent closing the vacuum chamber containing the plate on ice.
 - 2.3.2. Talent starting the vacuum pump to reach the desired pressure
 - 2.3.3. Talent slowly releasing the vacuum from the chamber.
 - 2.3.4. Talent mixing the sample in the plate and placing the plate back again in the

chamber.

- 2.4. Under the fume hood, remove the fixation solution with a pipette [1]. Wash each well 3 times for 10 minutes each under gentle agitation using 5 milliliters of ice-cold PBS [2].
 - 2.4.1. Talent removing the fixation solution from each well with a pipette under the fume hood.
 - 2.4.2. Talent placing the plate added with PBS under gentle agitation on a rocker.
- 2.5. After the washes, remove the PBS [1]. Using forceps, transfer the fixed tissue to a 120 by 120 millimeter Petri dish [2] and add 50 microliters of nuclei isolation buffer to the fixed seedlings [3].
 - 2.5.1. Talent removing PBS from the fixed tissue.
 - 2.5.2. Talent transferring the fixed tissue to a 120 millimeter by 120 millimeter Petri dish with forceps.
 - 2.5.3. Talent adding 50 µl of nuclei isolation buffer to the fixed seedlings.

3. Isolation of Nuclei from Fixed Seedlings

- 3.1. With a blade, chop the fixed tissue [1]. Using the same blade, scrape the chopped tissue to one edge of the Petri dish [2]. Add 600 microliters of nuclei isolation buffer and wash off any tissue stuck to the blade [3].
 - 3.1.1. Talent chopping the fixed tissue with a blade.
 - 3.1.2. Talent scraping the chopped tissue to one edge of the Petri dish with a blade.
 - 3.1.3. Talent adding nuclei isolation buffer to the dish while rinsing tissue from the blade.
- 3.2. Transfer the tissue slurry into a 2 milliliter Dounce homogenizer tube [1] and stroke 5 to 10 times with the smaller pestle [2]. Then, stroke with the larger pestle until it moves smoothly inside the tube, typically requiring 5 to 10 strokes [3].
 - 3.2.1. Talent transferring the tissue slurry into a 2 milliliter Dounce homogenizer tube.
 - 3.2.2. Talent homogenizing the sample with the smaller pestle.
 - 3.2.3. Talent homogenizing the sample with the larger pestle.

3.3. Now, filter the homogenized sample through a 30-micrometer mesh filter into a 50 milliliter tube [1]. Transfer the filtrate into a fresh 1.5-milliliter tube [2]. **NOTE: The VO is edited for the additional shot**

3.3.1. Talent filtering the homogenized sample through a mesh filter into a 50 ml tube.

Added shot: Talent transferring the sample from the 50 ml tube into a 1.5-milliliter microtube.

3.4. Centrifuge the suspension for 10 minutes at 800 *g* using the lowest ramp for both acceleration and deceleration [1]. After centrifugation, note that a small white pellet is visible among the green tissue debris [2].

3.4.1. Talent placing the microtube into the centrifuge.

3.4.2. Shot of a small white pellet visible among green tissue debris in the tube.
Authors: If possible, please prepare the end product of this sample

3.5. Discard the supernatant [1] and resuspend the pellet in 150 microliters of nuclei isolation buffer [2].

3.5.1. Talent removing and discarding the supernatant from the microtube.

3.5.2. Talent resuspending the pellet in nuclei isolation buffer by pipetting up and down.

3.6. Centrifuge again for 10 minutes at 800 *g* and remove the supernatant [1].

3.6.1. Talent placing the microtube into the centrifuge.

3.7. After completely washing off the green debris, store the isolated nuclei suspension at 4 degrees Celsius for a maximum of 1 week [1] and immuno-stain the nuclei with desired antibodies [2].

3.7.1. Talent placing the microtube containing nuclei suspension into cold storage.

3.7.2. Shot of the adding the antibody mix to the sample.

4. Sample Mounting in STORM Buffer and Agarose

- 4.1. Heat the 1.5% weight by volume low-melting agarose solution at 90 degrees Celsius for 1 minute to dissolve the powder and obtain a clear solution [1]. Cool the solution and maintain it at 37 degrees Celsius before proceeding to avoid heat denaturation of the glucose oxidase buffer [2].
 - 4.1.1. Talent placing the solution into a heating device and vortexing the tube.
 - 4.1.2. Talent placing the solution at 37 degrees Celsius.
- 4.2. After the low-melting agarose solution reaches 37 degrees Celsius, add 100 millimolar mercaptoethylamine and glucose oxidase to achieve a 1x final concentration and prepare the STORM (*storm*) buffer [1-TXT] and mix the solution gently [2].
 - 4.2.1. Talent adding mercaptoethylamine and glucose oxidase to the warm agarose solution. **TXT: STORM: Stochastic Optical Reconstruction Microscopy**
 - 4.2.2. Talent gently mixing the solution.
- 4.3. Deposit 10 microliters of the mixture onto an ozone-treated 1.5-H coverslip that has been treated for 20 minutes in an ultraviolet ozone cleaning system [1]. Wait for 3 minutes to allow the nuclei to deposit on the coverslip [2].
 - 4.3.1. Talent pipetting the sample onto a coverslip.
 - 4.3.2. Shot of the sample resting on the coverslip during deposition.
- 4.4. Then, remove the excess liquid and wait for 2 minutes [1]. Add 10 microliters of the prepared low-melting agarose to the coverslip and immediately cover with a microscope slide [2].
 - 4.4.1. Talent removing excess liquid from the coverslip.
 - 4.4.2. Talent adding agarose and placing a microscope slide over the coverslip.
- 4.5. Seal the coverslip using silicone sealant [1]. Mix equal amounts of solution A and solution B in a small Petri dish using a micropipette tip [2]. Apply the mixture over the edges of the coverslip to seal the sample and allow it to harden for 5 minutes [3].
 - 4.5.1. Talent applying silicone sealant around the coverslip.
 - 4.5.2. Talent mixing solution A and solution B in a Petri dish using a micropipette tip.
 - 4.5.3. Talent applying the sealant mixture along the edges of the coverslip.

5. STORM Image Acquisition and Reconstruction

- 5.1. Mount the sample slide on the microscope and find focus [1]. Locate and focus on a nucleus [2]. Activate a focus-maintaining module if available to ensure stability during long acquisitions [3].
 - 5.1.1. Talent placing the sample slide onto the microscope stage and adjusting focus.
 - 5.1.2. SCREEN:70891_screenshot_B1.mp4: 00:05-00:17
 - 5.1.3. Talent activating the focus-maintaining module.
- 5.2. Select a region where one or a few nanodiamonds are positioned near the nucleus to improve drift correction during reconstruction [1].
 - 5.2.1. SCREEN: 70891_screenshot_B1.mp4: 00:17-00:27
- 5.3. For two-color imaging, begin acquisition with the longer wavelength channel [1] and then switch to the shorter wavelength channel to complete imaging [2].
 - 5.3.1. SCREEN: 70891_screenshot_B2.mp4: 00:04-00:20
 - 5.3.2. SCREEN: : 70891_screenshot_B3.mp4: 00:03-00:17, 00:22-00:27
- 5.4. Process the raw image stacks in **Thunder-STORM** using Gaussian fitting with Weighted Least-Squares estimation and generate reconstructed images [1]. Apply filtering based on sigma and uncertainty below 30 nanometers to retain accurate localizations and remove noise [2].
 - 5.4.1. SCREEN: : 70891_screenshot_B4.mp4: 00:04-00:25
 - 5.4.2. SCREEN: : 70891_screenshot_B5.mp4: 00:06-end
- 5.5. Finally, perform sample drift correction using **ThunderSTORM** cross-correlation or the **COMET** (*comet*) algorithm [1] and confirm minimal drift in the reconstructed images [2].
 - 5.5.1. SCREEN: : 70891_screenshot_B6.mp4: 00:00-00:17
 - 5.5.2. SCREEN: : 70891_screenshot_B7.mp4: 00:04-00:08, 00:23-00:25

Results

6. Results

6.1. Nuclei were isolated from 15-day-old wild-type seedlings, immunolabeled with AF647-conjugated secondary antibodies, and counterstained with Hoechst H33258 (H-3-3-2-5-8) [1].

6.1.1. LAB MEDIA: Figure 1

6.2. Confocal imaging showed multiple isolated and well-preserved nuclei with minimal debris [1], with chromocenters and the nucleolus clearly distinguishable [2].

6.2.1. LAB MEDIA: Figure 1. *Video editor: Highlight Hoechst column images*

6.2.2. LAB MEDIA: Figure 1. *Video editor: Zoom in on the top image in Hoechst and AF647 columns and point to the regions indicated by arrows*

6.3. RNA Polymerase II (2) signal was absent from chromocenters and the nucleolus [1].

6.3.1. LAB MEDIA: Figure 1. *Video editor: Highlight the areas marked by arrows where the red signal is missing*

6.4. Diffraction-limited maximum intensity projections and corresponding SMLM reconstructions revealed structural details of RNA Polymerase II and DNA labeling [1], including enlarged regions of interest showing finer structures [2].

6.4.1. LAB MEDIA: Figure 2. *Video editor: Highlight the AF647 and JF549 columns images*

6.4.2. LAB MEDIA: Figure 2. *Video editor: Highlight the boxed inset regions after each image*

6.5. Localization filtering based on sigma and uncertainty improved the definition of both RNA Polymerase II and DNA signals compared to detected localizations [1], and further refinement after drift correction maintained this improved structure [2].

6.5.1. LAB MEDIA: Figure 3A. *Video editor: Show the top row labeled “detected localizations”*

6.5.2. LAB MEDIA: Figure 3A. *Video editor: Move to the middle and bottom rows and*

6.6. Filtering based on sigma values removed localizations with excessively small or large

fitted widths [1].

6.6.1. LAB MEDIA: Figure 3B.

6.7. Fourier Ring Correlation analysis showed that resolution in the AF647 channel improved from 90.1 to 47.7 nanometers after filtering [1], while the JF549 channel improved from 56.6 to 27.9 nanometers [2].

6.7.1. LAB MEDIA: *Figure 4. Video editor: Highlight the resolution value labeled 90.1 nanometer in panel A and then 47.7 nanometer in panel C for the left column*

6.7.2. LAB MEDIA: *Figure 4. Video editor: Highlight the resolution value labeled 56.6 nanometer in panel A and then 27.9 nanometer in panel C for the right column*

1. Formaldehyde
Pronunciation link: <https://www.merriam-webster.com/dictionary/formaldehyde>
IPA: /fər'mæl.dəˈhaɪd/
Phonetic Spelling: fer·mal·duh·hyde
2. Triton
Pronunciation link: <https://www.merriam-webster.com/dictionary/Triton>
IPA: /'traɪ.tə:n/
Phonetic Spelling: try·ton
3. Arabidopsis
Pronunciation link: <https://www.merriam-webster.com/dictionary/Arabidopsis>
IPA: /əˌræbrɪ'dɑːpsɪs/
Phonetic Spelling: uh·rab·ih·dop·sis
4. Germination
Pronunciation link: <https://www.merriam-webster.com/dictionary/germination>
IPA: /ˌdʒɜː.nə'meɪ.jən/
Phonetic Spelling: jur·muh·nay·shuhn
5. Murashige
Pronunciation link: No confirmed link found
IPA: /ˌmʊrə'ʃiːgeɪ/
Phonetic Spelling: moo·ruh·shee·gay
6. Vacuum
Pronunciation link: <https://www.merriam-webster.com/dictionary/vacuum>

- IPA: /'væk.ju:m/, /'væk.jəm/
Phonetic Spelling: vak·yoom, vak·yuhm
7. Fixative
Pronunciation link: <https://www.merriam-webster.com/dictionary/fixative>
IPA: /'fɪk.sə.tɪv/
Phonetic Spelling: fɪk·suh·tɪv
 8. Agitation
Pronunciation link: <https://www.merriam-webster.com/dictionary/agitation>
IPA: /,ædʒ.ə'teɪ.ʃən/
Phonetic Spelling: əj·uh·tay·shuhn
 9. Nuclei
Pronunciation link: <https://www.merriam-webster.com/dictionary/nuclei>
IPA: /'nu:.kli.əl/, /'nju:-/
Phonetic Spelling: noo·klee·eye
 10. Homogenizer
Pronunciation link: <https://www.merriam-webster.com/dictionary/homogenizer>
IPA: /hə'mɑ:.dʒə.naɪ.zə/
Phonetic Spelling: huh·mah·juh·ny·zer
 11. Pestle
Pronunciation link: <https://www.merriam-webster.com/dictionary/pestle>
IPA: /'pes.əl/
Phonetic Spelling: pes·uhl
 12. Filtrate
Pronunciation link: <https://www.merriam-webster.com/dictionary/filtrate>
IPA: /'fɪl.treɪt/
Phonetic Spelling: fɪl·trayt
 13. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /'sen.trəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
 14. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /,suː.pə'nei.tənt/
Phonetic Spelling: soo·per·nay·tuhnt
 15. Resuspend
Pronunciation link: <https://www.merriam-webster.com/dictionary/resuspend>
IPA: /,riː.sə'spend/
Phonetic Spelling: ree·suh·spend
 16. Agarose
Pronunciation link: <https://www.merriam-webster.com/dictionary/agarose>
IPA: /'æɡ.əˌroʊs/
Phonetic Spelling: əg·uh·rohs
 17. Denaturation
Pronunciation link: <https://www.merriam-webster.com/dictionary/denaturation>

- IPA: /diːˌneɪtʃəˈeɪ.ʃən/
Phonetic Spelling: dee·nay·chuh·ray·shuhn
18. Mercaptoethylamine
Pronunciation link: No confirmed link found
IPA: /mɜːˌkæp.tuːˌeθ.əlˈæm.iːn/
Phonetic Spelling: mer·kap·toh·eth·uhl·am·een
19. Microscopy
Pronunciation link: <https://www.merriam-webster.com/dictionary/microscopy>
IPA: /maɪˈkraɪˌskə.pi/
Phonetic Spelling: my·krah·skuh·pee
20. Nanodiamonds
Pronunciation link: No confirmed link found
IPA: /ˌnæ.noʊˈdaɪ.məndz/
Phonetic Spelling: nan·oh·die·muhndz
21. Reconstruction
Pronunciation link: <https://www.merriam-webster.com/dictionary/reconstruction>
IPA: /ˌriː.kənˈstrʌk.ʃən/
Phonetic Spelling: ree·kuhn·struk·shuhn
22. Gaussian
Pronunciation link: <https://www.merriam-webster.com/dictionary/Gaussian>
IPA: /ˈɡaʊ.si.ən/
Phonetic Spelling: gow·see·uhn
23. Localization
Pronunciation link: <https://www.merriam-webster.com/dictionary/localization>
IPA: /ˌloʊ.kə.ləˈzeɪ.ʃən/
Phonetic Spelling: loh·kuh·luh·zay·shuhn
24. Chromocenters
Pronunciation link: No confirmed link found
IPA: /ˈkroʊ.moʊˌsen.təz/
Phonetic Spelling: kroh·moh·sen·terz
25. Nucleolus
Pronunciation link: <https://www.merriam-webster.com/dictionary/nucleolus>
IPA: /nuːˈkliː.ə.ləs/, /njuː-/
Phonetic Spelling: noo·klee·uh·luhs
26. Polymerase
Pronunciation link: <https://www.merriam-webster.com/dictionary/polymerase>
IPA: /pəˈlɪm.əˌreɪs/
Phonetic Spelling: puh·lim·uh·rays
27. Diffraction
Pronunciation link: <https://www.merriam-webster.com/dictionary/diffraction>
IPA: /dɪˈfræk.ʃən/
Phonetic Spelling: dih·frak·shuhn
28. Fourier
Pronunciation link: <https://www.merriam-webster.com/dictionary/Fourier>

IPA: /'fɔr.i.eɪ/

Phonetic Spelling: foor·ee·ay

29. Correlation

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

IPA: /,kɔːr.ə'leɪ.ʃən/

Phonetic Spelling: kor·uh·lay·shuhn

30. Nanometers

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

IPA: /'næn.ə.mi:.tə/

Phonetic Spelling: nan·uh·mee·ter