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Project Page Link: <https://review.jove.com/account/file-uploader?src=21309788>

Title: A Multilabel Single Molecule Localization Microscopy Protocol for Investigation of Chromatin in the Dense Nuclear Environment

Authors and Affiliations:

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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? **NO**

- 3. Filming location:** Will the filming need to take place in multiple locations? **YES**
3 floors apart. Basement lab and 4th floor conference room

- 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: 24

Number of Shots: 55

Introduction

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

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**.

INTRODUCTION:

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

- 1.1. **Nicolas Acosta:** Our research examines the epigenetic composition of chromatin packing domains and how regulatory factors shape their structure and function.
 - 1.1.1. 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.~~

- 1.2. **Nicolas Acosta:** This protocol is most useful for investigating spatial relationships between targets in dense cellular environments, such as the nucleus.
 - 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?~~

- 1.4. **Nicolas Acosta:** Using this protocol, researchers can interrogate the seemingly disordered organization of chromatin to explore the relationships between its structure, regulatory elements, and functional outcomes.
 - 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. **Nicolas Acosta:** Following this procedure, various image-based or cloud computational analyses can extract quantitative structural features from spatial data, depending on the imaging modality used.

- 1.5.1. 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.~~

- 1.6. **Nicolas Acosta:** Researchers can extend this labeling method by optimizing additional markers and leveraging multi-channel data to enable more advanced and comprehensive analyses.

- 1.6.1. 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.

- Testimonial statements will **not appear in the video** but may be featured in the journal's promotional materials.
- **Provide the full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author delivering the testimonial.
- Please **answer the testimonial question live during the shoot**, speaking naturally and in your own words in **complete sentences**.

What problem in your field of research does your article address?

- 1.7. **Nicolas Acosta Cortes, PhD Candidate, Biomedical Engineering:** (authors will present their testimonial statements live)

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

How does the video format of your article help you to share your work with the broader scientific community?

- 1.8. **Nicolas Acosta Cortes, PhD Candidate, Biomedical Engineering:** (authors will present their testimonial statements live)

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

Protocol

2. Preparation of Bovine Serum Albumin Blocking Buffer

Demonstrator: Nicolas Acosta

2.4. To begin, weigh bovine serum albumin so that the final concentration in the required buffer volume for the experiment is 3 percent [1]. Add the bovine serum albumin to a centrifuge tube [2].

2.4.1. WIDE: Talent standing at the laboratory bench weighing bovine serum albumin on a balance.

2.4.2. Talent adding the weighed bovine serum albumin into a centrifuge tube.

2.5. Tilt the centrifuge tube to a 45-degree angle to spread the bovine serum albumin crystals along the tube wall [1]. Add PBS to the tube to prevent crystal clumping [2].

2.5.1. Talent tilting the centrifuge tube to approximately 45 degrees, so the bovine serum albumin crystals spread along the wall. NOTE: 2.5.1 and 2.5.2 are combined in a single shot

2.5.2. Talent adding PBS into the tilted centrifuge tube.

2.6. Allow all bovine serum albumin crystals to dissolve at room temperature [1-TXT]. Once the crystals are fully dissolved, add Triton X-100 to achieve a final concentration of 0.2% [2]. If crystals remain, pipette the solution up and down several times to mix the solution slowly without forming bubbles [3].

2.6.1. Talent placing the centrifuge tube containing the solution in a rack on the working area. TXT: Avoid shaking or vortexing the tube NOTE: 2.6.1 is included with 2.5.2

2.6.2. Talent adding Triton X-100 into the centrifuge tube containing the dissolved bovine serum albumin solution.

2.6.3. Talent gently pipetting the solution up and down inside the centrifuge tube.

3. Immunofluorescence Cell Staining Procedure

3.4. Take the live cells from the incubator [1]. Remove the cell culture medium from the dish [2-TXT].

3.4.1. WIDE: Talent opening the incubator and removing a dish containing live cells.

TXT: BJ fibroblast, HCT116, AC16, HeLa, MCF10A can be used for staining

- 3.4.2. Talent pipettes the cell culture medium from the dish. **TXT: Discard medium into a biohazard waste container** **NOTE: 3.4.2 to 3.6.2 are filmed in a single shot**
- 3.5. Now add enough PBS to cover the cells [1]. Gently swirl the dish to wash the cells [2] and then pipette out the PBS [3].
- 3.5.1. Talent pipetting PBS into the dish until the cell surface is covered.
- 3.5.2. Talent gently swirling the dish to wash the cells.
- 3.5.3. Talent pipettes the PBS from the dish.
- 3.6. Next, pipette enough fixative solution to cover the cells [1] and leave the cells to fix for 10 minutes [2].
- 3.6.1. Talent pipetting fixative solution into the dish to fully cover the cells.
- 3.6.2. Talent places the dish aside on the bench to allow fixation.
- 3.7. Using a balance, weigh sodium borohydride for the quenching solution [1-TXT]. Add the sodium borohydride to a centrifuge tube [2].
- 3.7.1. Talent weighing sodium borohydride on a balance. **TXT: Final Concentration: 0.1% (w/v) solution**
- 3.7.2. Talent transferring the sodium borohydride into a centrifuge tube.
- 3.8. Pipette out the fixative solution from the dish [1]. Then add enough PBS to the dish to cover the cell surface [2]. Place the dish on a shaker for 5 minutes to wash the cells [3].
- 3.8.1. Talent aspirating the fixative solution from the dish. **NOTE: 3.8.1 and 3.8.2 are filmed in a single shot**
- 3.8.2. Talent pipetting PBS into the dish.
- 3.8.3. Talent placing the dish onto a laboratory shaker.
- 3.9. Now add the required volume of PBS to the sodium borohydride in the centrifuge tube [1-TXT]. Vortex the tube to mix the quenching solution [2].
- 3.9.1. Talent pipetting PBS into the centrifuge tube containing sodium borohydride. **TXT: Final concentration: 0.1% (w/v); Adjust the volume as per the requirement** **Added shot - 3.7.2.1: adding PBS to tube, but realized that it was**

3.9.1 later. So 3.9.1 is slated as 3.7.2.1

3.9.2. Talent vortexing the centrifuge tube.

3.10. Remove the dish from the shaker [1] and discard the PBS from the dish [2].

3.10.1. Talent removing the dish from the shaker.

3.10.2. Talent aspirating PBS from the dish. NOTE: 3.10.2-3.11.1 are filmed in a single shot

3.11. Add enough quenching solution to cover the surface of the dish [1]. Then place the dish on a shaker for 7 minutes to quench autofluorescence in the cells [2]. Discard the remaining quenching solution in the centrifuge tube into the appropriately labeled liquid chemical waste container [3].

3.11.1. Talent pipetting quenching solution into the dish to cover the cell surface.

3.11.2. Talent placing the dish onto the shaker. NOTE: Reuse 3.8.3

3.11.3. Talent pouring the remaining quenching solution from the centrifuge tube into a labeled liquid chemical waste container.

3.12. Now, remove the dish from the shaker [1] and remove the quenching solution from the dish [2].

3.12.1. Talent removing the dish from the shaker. NOTE: Reuse 3.10.1

3.12.2. Talent aspirating the quenching solution from the dish. NOTE: Reuse 3.10.2

3.13. Then add enough PBS to the dish to cover the surface [1]. Place the dish on a shaker for 5 minutes to wash the cells [2]. After incubation, remove the PBS and repeat the wash with PBS two additional times [3].

3.13.1. Talent pipetting PBS into the dish.

3.13.2. Talent placing the dish onto the shaker.

3.13.3. Talent aspirating PBS from the dish.

3.14. Now add enough blocking buffer to the dish to cover the surface [1]. Incubate the dish on a shaker for at least 1 hour to permeabilize the cell membranes and block binding sites [2].

3.14.1. Talent pipetting blocking buffer into the dish to fully cover the cells.

3.14.2. Talent placing the dish onto the shaker.

3.15. Prepare the primary antibody staining solution according to the recommended concentration [1-TXT]. Remove the required volume from the blocking buffer stock [2]. Transfer this volume to a new centrifuge tube to prepare the staining solution [3].

3.15.1. Talent preparing reagents for the primary antibody staining solution at the bench. **TXT: Calculate total staining volume by adding 0.5 mL to the volume required for cell coverage** **NOTE: 3.15.1 also has 3.15.2, and 3.15.3, and 3.16.1 included.**

3.15.2. Talent pipetting blocking buffer from a stock container.

3.15.3. Talent transferring the measured blocking buffer into a new centrifuge tube.

3.16. Add the required volume of primary antibody stock to the blocking buffer to achieve the final concentration specified by the manufacturer [1-TXT].

3.16.1. Talent pipetting primary antibody stock into the centrifuge tube containing blocking buffer. **TXT: Common dilutions: 1:1000, 1:500, or 1:100**

3.17. Now, replace the blocking buffer with enough primary antibody staining solution to cover the surface of the dish [1-TXT].

3.17.1. Talent pipetting primary antibody staining solution into the dish. **TXT: Incubate on a shaker for 1–2 hours or up to O/N**

3.18. After incubation with the primary antibody, replace the primary antibody solution with washing buffer [1]. Then place the dish on a shaker for 5 minutes to wash the cells [2]. Repeat the washing buffer wash two additional times for a total of 3 washes [3].

3.18.1. Talent pipetting washing buffer into the dish.

3.18.2. Talent placing the dish onto the shaker. **NOTE: reuse 3.14.2**

3.18.3. Talent repeating the washing buffer washing. **NOTE: Reuse 3.13.3**

3.19. Prepare the secondary antibody staining solution according to the recommended concentration [1]. Calculate the total staining solution volume by adding 0.5 milliliters to the volume required to cover the cells [2].

3.19.1. Talent preparing reagents for the secondary antibody staining solution at the bench. **NOTE: 3.19.1 - 3.21.1 filmed in a single shot**

3.19.2. Talent calculating the required staining solution volume.

3.20. Transfer the calculated volume from the blocking buffer into a new centrifuge tube to prepare the staining solution [1]. Then add the appropriate volume of secondary antibody stock to the blocking buffer to obtain the correct final concentration [2].

3.20.1. Talent transferring the blocking buffer into a new centrifuge tube.

3.20.2. Talent pipetting secondary antibody stock into the centrifuge tube.

3.21. Wrap the centrifuge tube containing the secondary antibody staining solution with aluminum foil until ready for use [1].

3.21.1. Talent wrapping the centrifuge tube with aluminum foil.

3.22. Add enough secondary antibody staining solution to the dish to cover the surface [1]. Place the dish on a shaker for 40 minutes to attach fluorophores to the labeled cellular targets [2]. Cover the dish with aluminum foil to prevent fluorophore bleaching [3].

3.22.1. Talent pipetting secondary antibody staining solution into the dish. NOTE: First take of 3.22.1 removes the last washing buffer before adding the secondary antibody.

3.22.2. Talent placing the dish onto the shaker.

3.22.3. Talent covering the dish with aluminum foil. NOTE: 3.22.3 included in 3.22.2

3.23. After 40 minutes, perform 2 PBS washes [1] as demonstrated earlier [2]. Then add enough PBS to the dish to cover the cell surface before storage [2].

3.23.1. Talent pipetting PBS into the dish.

3.23.2. Talent placing the dish onto the shaker.

3.23.3. Talent pipetting PBS into the dish.

3.24. Wrap the dish with parafilm to prevent liquid evaporation [1] and then with aluminium foil to prevent fluorophore bleaching [2]. Store the wrapped dish at 4 degrees Celsius until ready to image [3-TXT].

3.24.1. Talent sealing the dish with parafilm.

3.24.2. Talent wrapping the sealed dish with aluminum foil.

3.24.3. Talent placing the wrapped dish into a refrigerator set to 4 degrees Celsius. TXT:

Similarly, process each label using modified blocking and washing buffers

Results

4. Results

4.4. The sequential staining protocol produced representative three-color chromatin dSTORM (*D-Storm*) images for multiple cell lines, including BJ (*B-J*) fibroblast [1], HeLa (*He-Lah*) [2], and MCF10A (*M-C-F-Ten-A*) cells [3].

4.4.1. LAB MEDIA: Figure 1A.

4.4.2. LAB MEDIA: Figure 1B.

4.4.3. LAB MEDIA: Figure 1C.

4.5. The analysis pipeline was evaluated using simulated datasets representing normal, uniform, toroidal, and random spatial distributions anchored to heterochromatin cluster positions [1].

4.5.1. LAB MEDIA: Figure 2C. *Video editor: Show the two plots titled “Normal Toroidal” and “Normal - Random.”*

4.6. Distinct spatial organization patterns produced characteristic distance histogram profiles when localization coordinates were analyzed relative to heterochromatin centroids [1].

4.6.1. LAB MEDIA: Figure 2D. *Video editor: Show the three histograms*

4.7. Simulated spatially segregated markers in a normal-toroidal configuration produced minimal joint density, resulting in a flat distribution profile [1]. Simulated overlapping marker patterns in a normal-random configuration produced decreasing joint density with increasing distance from reference points [2].

4.7.1. LAB MEDIA: Figure 2E. *Video editor: Highlight the blue curve labeled “Toroidal Marker 2.”*

4.7.2. LAB MEDIA: Figure 2E. *Video editor: Highlight the orange curve labeled “Random Marker 2.”*

4.8. DBSCAN-based identification of heterochromatin domains enabled categorization into small, medium, and large domains based on effective radius [1]. Quantitative distance

analysis showed that RNA Polymerase II localized near heterochromatin boundaries across small, medium, and large domains [2].

4.8.1. LAB MEDIA: Figure 3A.

4.8.2. LAB MEDIA: Figure 3A. *Video editor: Highlight the central peaks of the three histograms*

4.9. Quantitative distance analysis showed that H3K27ac (*H-Three-K-Twenty-Seven-A-C*) localized near heterochromatin cluster boundaries across small, medium, and large domains [1].

4.9.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the central peaks of the three histograms labeled "Small," "Medium," and "Large."*

4.10. Joint density analysis showed peak H3K27ac and RNA Polymerase II (*Two*) co-localization just outside heterochromatin cluster boundaries [1].

4.10.1. LAB MEDIA: Figure 3I. *Video editor: Highlight the tallest peaks in the graph titled "Joint Density RNAP II/H3K27ac."*

1. Bovine serum albumin
Pronunciation link: <https://www.merriam-webster.com/dictionary/albumin>
IPA: /æɪˈbjʊː.mɪn/
Phonetic Spelling: al·byoo·min
2. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsɛn.trɪ.fjuː.dʒ/
Phonetic Spelling: sen·truh·fyooj
3. Phosphate-buffered saline (PBS)
Pronunciation link: <https://www.merriam-webster.com/dictionary/phosphate>
IPA: /ˈfɑːs.fɛt/
Phonetic Spelling: faas·fayt
4. Triton X-100
Pronunciation link: No confirmed link found
IPA: /ˈtraɪ.tɑːn/
Phonetic Spelling: try·ton

5. Immunofluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/immunofluorescence>
IPA: /ɪˌmjuː.nəʊ.fluˈres.əns/
Phonetic Spelling: ih·myoo·noh·floor·es·uhns
6. Fibroblast
Pronunciation link: <https://www.merriam-webster.com/dictionary/fibroblast>
IPA: /ˈfaɪ.brəˌblæst/
Phonetic Spelling: fy·bruuh·blast
7. Fixative
Pronunciation link: <https://www.merriam-webster.com/dictionary/fixative>
IPA: /ˈfɪk.sə.tɪv/
Phonetic Spelling: fik·suh·tiv
8. Sodium borohydride
Pronunciation link: <https://www.merriam-webster.com/dictionary/borohydride>
IPA: /ˌbɔːr.oʊˈhaɪ.draɪd/
Phonetic Spelling: bor·oh·hy·dryd
9. Quenching
Pronunciation link: <https://www.merriam-webster.com/dictionary/quench>
IPA: /kwentʃ/
Phonetic Spelling: kwench
10. Autofluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/autofluorescence>
IPA: /ˌɔː.təʊ.fluˈres.əns/
Phonetic Spelling: aw·toh·floor·es·uhns
11. Permeabilize
Pronunciation link: <https://www.merriam-webster.com/dictionary/permeabilize>
IPA: /ˈpɜː.mi.ə.bəˌlaɪz/
Phonetic Spelling: per·mee·uh·buh·lyz
12. Antibody
Pronunciation link: <https://www.merriam-webster.com/dictionary/antibody>
IPA: /ˈæn.tɪˌbɑː.di/
Phonetic Spelling: an·tee·baa·dee
13. Fluorophore
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorophore>
IPA: /ˈflɒr.əˌfɔːr/
Phonetic Spelling: floor·uh·for
14. Parafilm
Pronunciation link: No confirmed link found
IPA: /ˈpær.ə.fɪlm/
Phonetic Spelling: pair·uh·film
15. Chromatin
Pronunciation link: <https://www.merriam-webster.com/dictionary/chromatin>
IPA: /ˈkroʊ.mə.tɪn/
Phonetic Spelling: kroh·muh·tin

16. Toroidal
Pronunciation link: <https://www.merriam-webster.com/dictionary/toroidal>
IPA: /təˈrɔɪ.dəl/
Phonetic Spelling: tuh·roy·duhl
17. Heterochromatin
Pronunciation link: <https://www.merriam-webster.com/dictionary/heterochromatin>
IPA: /ˌhet.ə.roʊˈkroʊ.mə.tɪn/
Phonetic Spelling: het·uh·roh·kroh·muh·tin
18. Localization
Pronunciation link: <https://www.merriam-webster.com/dictionary/localization>
IPA: /ˌloʊ.kə.ləˈzeɪ.ʃən/
Phonetic Spelling: loh·kuh·luh·zay·shuhn
19. Centroid
Pronunciation link: <https://www.merriam-webster.com/dictionary/centroid>
IPA: /ˈsen.trɔɪd/
Phonetic Spelling: sen·troyd
20. DBSCAN
Pronunciation link: No confirmed link found
IPA: /ˈdiː.biː.skæn/
Phonetic Spelling: dee·bee·skan
21. Polymerase
Pronunciation link: <https://www.merriam-webster.com/dictionary/polymerase>
IPA: /pəˈlɪm.əˌreɪs/
Phonetic Spelling: puh·lim·uh·rays
22. Co-localization
Pronunciation link: <https://www.merriam-webster.com/dictionary/colocalization>
IPA: /ˌkoʊ.loʊ.kə.ləˈzeɪ.ʃən/
Phonetic Spelling: koh·loh·kuh·luh·zay·shuhn
23. Histogram
Pronunciation link: <https://www.merriam-webster.com/dictionary/histogram>
IPA: /ˈhɪs.təˌɡræm/
Phonetic Spelling: his·tuh·gram