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Title: Mapping Absolute DNA Density in Cell Nuclei Using Single-Molecule Localization Microscopy

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Author Questionnaire

1. **Microscopy**: Does your protocol demonstrate the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? No

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **yes**

3. **Proposed filming date:** To help JoVE process and publish your video in a timely manner, please indicate the proposed date that your group will film here: **MM/DD/YYYY**
DO NOT use this draft script for filming. Please wait until your script is finalized to begin the filming process.

When you are ready to submit your video files, please contact our Content Engineer, Devon Halley.

Current Protocol Length

Number of Steps: 33
Number of Shots: 56

Introduction

NOTE: This entire project was filmed from the draft script, and the script was never finalized. It omitted the finalization stage and hence, the red header about not using it for filming.

1.1. **Marton Gelleri**: The present method allows absolute DNA density measurements in cell nuclei to investigate the spatial constraints in chromatin structures that are otherwise only characterized by post-translational histone modifications. **[1]**.

1.1.1. LAB MEDIA: 1.1.1 DSC_2711, 00:00-00:12

What advantage does your protocol offer compared to other techniques?

1.2. **Hilmar Strickfaden**: Voronoi tessellation combined with SMLM allows absolute density estimations of DNA in terms of bp/µm^3 . Only a priori knowledge needed is the total DNA content of the measured cell nucleus. **[1]**.

1.2.1. LAB MEDIA: 1.2.1 DSC_2730, 00:00- 00:16.

What new scientific questions have your results paved the way for?

1.3. **Hilmar Strickfaden**: In future experiments, it would be interesting to investigate, whether absolute density differences correlate with biological information from other methods, such as immunofluorescence of epigenetic modifications or Hi-C data. **[1]**.

1.3.1. LAB MEDIA: 7.2 DSC_2710 -full clip

Protocol

Video editor: Screen captures have been added by Swati. If you have any queries, please contact her!

2. Cytometric Cell Cycle Determination

Demonstrator: Márton Gelléri

2.1. Begin by reconstructing the scanned area **[1]** by tiling the pre-recorded single images together **[2]**.

2.1.1. WIDE: Talent setting up the computer system with the screen visible in the screen. File name and timestamps: 2.1.1 - DSC_2706, 00:00 – 00:20.

2.1.2. SCREEN: 2.1.2 – Merging.m4v: 0:15-00:24, 01:22-01:26, and 01:33 – 01:35
Video editor: Please speed up 0:15-00:24

2.2. Open CellProfiler and then load the pipeline **CellCycleAnalysis.cpproj** *(Cell-cycle-analysis-dot-C-P-proj)* **[1]**. In the first step, **Images** of the pipeline, drag and drop the images, including the reference image **[2]**. Next, define a location where the reference images will be stored **[3]**, then click on the **Start Test Mode** icon followed by **Run [4]**.
NOTE: Narration has been edited!

2.2.1. SCREEN: CellCycleAnalysis: 00:00-00:09
2.2.2. SCREEN: CellCycleAnalysis: 00:09-00:16
2.2.3. SCREEN: CellCycleAnalysis: 00:16-00:24
2.2.4. SCREEN: CellCycleAnalysis: 00:16-00:31

2.3. Once the histogram of the nuclei in the image to be analyzed is displayed, determine the intensity intervals for the G1, S, and G2 phases **[1]** and make sure that they are entered in the subsequent "FilterObjects" steps of the pipeline and that these steps are active **[2]**. Then, click the **RUN** button again to continue with the second half of the pipeline **[3].**

2.3.1. SCREEN: CellProfilerCellCycleAnalysis: 01:03 - 01:17
2.3.2. SCREEN: CellProfilerCellCycleAnalysis: 01:17 - 01:32
2.3.3. SCREEN: CellProfilerCellCycleAnalysis 01:32 – 01:37

2.4. Look at the three windows overlaying the main cell cycle stages and zoom into one to see the different overlayed nuclei for a certain cell cycle stage **[1]**.

2.4.1. SCREEN: CellProfilerCellCycleAnalysis 02:32 - 02:40

2.5. Relocate the cells on the single-molecule localization microscope and ensure proper blinking of the fBALM *(F-balm)* process **[1]**.

2.5.1. Talent relocates one of the chosen cells on the the single-molecular localization microscope. File name and timestamps: 3.1.1 wide ensure blinking, 00:00-00:10

3. SMLM and fBALM Image Acquisition and Data Processing

3.1. Begin the image series acquisition using an exposure time of 50 milliseconds, resulting in a frame rate of approximately 20 frames per second **[1]**.

3.1.1. SCREEN: 3.1.2 - 3.2.2 Blinking 2: 00:21 – 00:29 *Video editor: Please emphasize "Exposure Time" and its value 50 ms in the box next to it. Its on top panel of screen*

3.2. Take a Z *(Zee)*-stack through the nucleus, with 200-nanometer step intervals and only 500 frames per light optical section **[1]**.

3.2.1. SCREEN: 3.1.2 - 3.2.2 Blinking 2: 00:30 – 00:36 *Video editor: On the left side panel, in the "Localization Mode" window, please emphasize "Step Size" and its value 200 [nm] in the box next to it. Then emphasize "Frames" and its value 500 in the box next to it.*

3.3. Open the f-BALM dataset in ImageJ *(Image-J)* **[1]**. To optimize the settings for the detection of blinking signals, click on **ThunderSTORM** *(thunder-storm)*, then **Run Analysis [2]**. Now, choose **Camera Setup**, enter the correct **pixel size** of the image data, the **A/D** *(A-by-D)* **count**, **Quantum efficiency** of the used camera, **base level**, and **EM** *(E-M)* **gain,** then click **OK [3].** **NOTE:** Narration is edited based on visuals.

3.3.1. SCREEN: 4.2-4.5 Thunderstorm_n01_: 00:00-00:03 and 00:13 – 00:16

3.3.2. SCREEN: 4.2-4.5 Thunderstorm_n01_: 00:24 – 00:30

3.3.3. SCREEN: 4.2-4.5 Thunderstorm_n01_: 00:31 – 00:40 *Video editor: In the "Camera setup" window that opens as a pop up, please emphasize each field and its value as VO speaks.*

3.4. Choose the algorithm to determine the localization coordinates by fitting the blinking signals. In the **Image Filtering** section, use the **Wavelet filter (B-Spline)** *(Wavelet filter B-Spline)* with a **B-Spline order 3** and a **B-Spline scale 2.0** *(two)* **[1]**.

3.4.1. SCREEN: 4.2-4.5 Thunderstorm_n01_: 00:41 – 00:48 *Video editor: Emphasize the "Image Filtering" section, then as VO says, emphasize "Filter" and its box containing "Wavelet filter (B-Spline)", followed by "B-Spline order" and its box with "3" value and a "B-Spline scale" and its box with "2.0" value*

3.5. Then, set the **Approximate localization of molecules method** to **Local maximum**, set the **Peak intensity threshold**, and **Connectivity** to **8-neighborhood** *(Eight neighborhood)* **[1-TXT]**.

3.5.1. SCREEN: 4.2-4.5 Thunderstorm_n01_00:49 - 00:55 *Video editor: Emphasize the "Approximate localization of molecules" section, then as VO says, emphasize "method" and its box containing "Local maximum", followed by "Peak intensity threshold" and its box with "2*std(Wave.F1)" and then, "Connectivity" and "8-neighborhood"* **TXT: Peak intensity threshold: 2*std(Wave.F1)**

3.6. In the **Sub-pixel localization of molecules** section, as a method, choose **PSF: Integrated Gaussian** *(P-S-F Integrated Gaussian)*, set **Fitting radius [px]** *(p-x)* to **3**, select **Fitting method** as **Maximum likelihood**, and set **Initial sigma [px]** to **1.6 [1]**. Then, click **OK** to start the detection of the signals and reconstruction of the image **[2].** **NOTE:** Narration is edited based on visuals.

3.6.1. SCREEN: 4.2-4.5 Thunderstorm_n01_00:56 - 01:12 *Video editor: Emphasize the "Sub-pixel localization of molecules" section, then as VO says, emphasize "method" and its box containing "PSF: Integrated Gaussian", followed by "Fitting radius [px]" and its box with "3" value, then, "Fitting method" and its box with "Maximum likelihood", and at last, "Initial sigma [px]" and its box with "1.6" value.*

3.6.2. SCREEN: 4.2-4.5 Thunderstorm_n01_01:23 - 01:27 and 04:35 – 04:38

3.7. To partition the acquisition data into multiple image stacks, begin concatenating the localization tables in ThunderSTORM **[1]**. To do so, click **Import,** in the pop-up window, ensure that the **Append to current table** option is activated and the correct starting number is used to avoid overwriting localizations in the table **[2]**. Then, select the file path and click **OK** to import one file after another **[3]**. NOTE: VO has been edited based on the visuals.

3.7.1. SCREEN: 4.6 - 4.7.2 Thunderstorm_n02: 00:00 – 00:05

3.7.2. SCREEN: 4.6 - 4.7.2 Thunderstorm_n02: 00:06 – 00:18 *Video editor: Emphasize the "Import pop-up window" and then as VO says emphasize "Append to current table" and the box next to it which is checked. When Vo talks about starting number, emphasize the "starting frame number section and its box in which 5001 is being typed.*

3.7.3. SCREEN: 4.6 - 4.7.2 Thunderstorm_n02: 00:23 – 00:29 and 01:00 – 01:07

3.8. To prevent overcounting signals across consecutive frames, merge the localization data using **Maximum Distance** of a 20 nanometer, **1 Maximum off frames**, and **0 Max.** *(maximum)* **frames per molecule.** Then, click **Merge** **[1].** NOTE: VO has been edited based on the visuals.

3.8.1. SCREEN: 4.8-4.9 Thunderstorm_n03_2: 00:00 – 00:17 *Video editor: Emphasize the bottom panel at the end of table where the settings are done and then as VO says emphasize "Maximum Distance [units of x, y]" and its value "20" ,*

"Maximum off frames" and its value "1", and "Max. frames per molecule (0 = unlimited)" and its value "0".

3.9. To measure and correct drift by cross-correlating data subsections, open the **Drift Correction** menu, click the **>>** *(arrows)*. Add 3 bins, set the **Magnification** to 5. Then, click **Apply,** and the window showing the x and y drift will appear **[1-TXT]**. NOTE: VO has been edited based on the visuals.

3.9.1. SCREEN: 4.8-4.9 Thunderstorm_n03_2: 00:18 - 00:32 and 00:50 – 00:53 **TXT: If analyzing a thin optical section, exclude all localizations outside the focal plane** *Video editor: Emphasize the bottom panel at the end of table where the settings are done and then as VO says emphasize "Number of bins" and its value "3", then, "Magnification" and its value "5.0" When VO says "the window showing the x and y drift will appear" show 00:50 – 00:53*

3.10. To determine DNA fraction in the pre-recorded section, choosing **plug-ins,** then **ThunderSTORM** followed by **Import/Export** *(import-export)* and **Import Results** for opening the first localization results table of the stack **[1]**. Press **Plot histogram** and in the opened Distribution dialogue, choose **z**, and press **ok [2].**

3.10.1. SCREEN: 4.10 Thunderstorm_n05_200: 00:00 – 00:13

3.10.2. SCREEN: 4.10 Thunderstorm_n05_200: 00:14 – 00:20

3.11. Determine the position of the peak and use the **Filter** field to select the signals with the optical step-size of 200 nm **[1-TXT].** Click on **Visualization** and choose the **Histograms** option, then click **OK [2]**. Save the resulting image in a folder in the TIFF *(tiff)* format **[3]**.

3.11.1. SCREEN: 4.10 Thunderstorm_n05_200: 00:25 – 00:32 **TXT: z >138 -100 & z < 138 + 100** *Video editor: Emphasize the bottom panel at the end of table where the settings are done*

3.11.2. SCREEN: 4.10 Thunderstorm_n05_200: 00:33 – 00:39

3.11.3. SCREEN: 4.10 Thunderstorm_n05_200: 00:42 – 01:07 *Video editor: Please speed up!*

3.12. Open all slices and combine them using **Image**, followed by **Stacks,** and then **Images to Stack [1]**. After selecting the whole image area, go to **Analyze**, click **Tools**, and select the **ROI Manager [2]**. Click on **Add**, then click on **Analyze**, followed by **Set Measurements** and select **Integrated Density [3]**. Now, choose the **More >>** *(more)* option, select **Multi Measure**, check **Measure all Stacks** as well as **One row per slice.** Click **OK,** and the results will appear **[4-TXT]**. NOTE: VO has been edited based on the visuals.

3.12.1. SCREEN: 4.11 Thunderstorm_n06_200: 00:00 - 00:14

3.12.2. SCREEN: 4.11 Thunderstorm_n06_200: 00:17 - 00:31

3.12.3. SCREEN: 4.11 Thunderstorm_n06_200: 00:38 - 00:48

3.12.4. SCREEN: 4.11 Thunderstorm_n06_200: 00:49 - 01:01 **TXT: Save the results and add them to get the integrated Density of all slides**

3.13. After opening the results table of the center plane, filter it to a thickness of 100-nanometer **[1-TXT].** Create the Histogram of the resulting signals as demonstrated earlier and measure the integrated density of the nucleus **[2]**.

3.13.1. SCREEN: 4.12 Thunderstorm_n07_100: 00:19 – 00:21 and 00:33 – 00:39 **TXT: z > 59-50 & z < 59 + 50**

3.13.2. SCREEN: 4.12 Thunderstorm_n07_100: 00:41 – 00:47 and 00:53 – 00:57

4. Voronoi Tessellation

4.1. Convert the **ThunderSTORM** localization table from the .csv *(dot-C-S-V)* format **[1]** into the **Orte** format by running the MATLAB *(mat-lab)* script TS2Orte.m *(T-S-two-orte-dot-M)*, which transforms the localization table into a MATLAB matrix and saves it in the .mat *(dot-mat)* format **[2]**.

4.1.1. SCREEN: 51_Excel: 00:37 – 00:42

4.1.2. SCREEN: 51_ThunderSTORM: 00:04 – 00:09, 00:23 - 00:31, 00:58 – 01:02, and 01:08 – 01:19

4.2. Go into the **LAND** *(land)*-**Voronoi** folder, and in the **coreAlgorithm** *(core-algorithm)* subfolder, open the file **voronoiCluster.m** *(vo-row-no-ee-cluster-dot-m)* and adjust the DNAcontent *(D-N-A content)*, **nLocalizations** *(N-localizations)*, **fractionDNA** *(Fraction D-N-A),* and conversion factor for the absolute density calculations **[1]**. NOTE: VO has been edited based on the visuals.

4.2.1. SCREEN: 53-55_LAND-Voronoi: 00:04 – 00:20 and 00:25 – 00:56

4.3. Edit the script that starts the analysis VonoRoi.m *(vo-no-roi-dot-m)*. Adjust the file path to the **Orte** localization data and the output folder for the result files **[1]**. Specify the coordinates defining the area in which the tessellation should be done. Define multiple areas to compute within the same input dataset **[2]**.

4.3.1. SCREEN: 53-55_LAND-Voronoi 01:08-2:00 *Video editor: For 4.3.1 and 4.3.2, if possible to speed up entire range, do it. If not, select first as well as last 10 to 15 seconds and speed them up!*

4.3.2. SCREEN: 53-55_LAND-Voronoi 2:00-4:00

4.4. After running the script, look for the image showing the absolute DNA densities together **[1]** with other files containing graphs showing the histogram of the area distribution **[2]** and a densities.mat *(densities-dot-m)* file containing the densities of every single calculated Voronoi-cell in the output folder **[1]**.

4.4.1. SCREEN: 53-55_LAND-Voronoi 09:31 – 09:40

4.4.2. SCREEN: 53-55_LAND-Voronoi 09:41 – 09:53

4.4.3. SCREEN: 53-55_LAND-Voronoi 09:58 – 10:09

Results

5. Results

5.1. The fBALM measurement consisting of 50,000 frames resulted in 2.68 × 10^6 detected localizations within a 100-nanometer thick midsection. The localization accuracy of the reconstructed image was 10 nanometers **[1].**

5.1.1. LAB MEDIA: Figure 5A.

5.2. The large number of localizations allowed the combination of super-resolution imaging while showing absolute DNA densities. It was apparent that the measured DNA densities were not uniformly distributed across the nucleus and covered an extensive dynamic range **[1].**

5.2.1. LAB MEDIA: Figure 5B.

5.3. Higher magnifications of areas within the nucleus showing larger Voronoi cells indicated low DNA densities, while smaller cells indicated high DNA densities **[1]**.

5.3.1. LAB MEDIA: Figures 5C', 5D' and 5E'. *Video editor: Emphasize the yellow boxes in C' and D'.*

5.4. DNA densities measured in a G_1 C3H10T1/2 *(see three 10 tee half)* nucleus showed the absolute DNA density distribution within the nucleus of a different type and species **[1]**. Although the basic organization looked like the early G_1 HeLa *(hela)* nucleus, it additionally possessed constitutive clusters of pericentric heterochromatin **[2]**.

5.4.1. LAB MEDIA: Figure 6. *Video editor: Emphasize C and D.*

5.4.2. LAB MEDIA: Figure 6.

5.5. No dramatic architectural changes was observed in a G_2 nucleus despite a slightly increased nuclear size **[1]**.

5.5.1. LAB MEDIA: Supplemental Figure S2.

5.6. The HeLa nucleus treated with TSA *(T-S-A)* showed remarkable differences with respect to nuclear topography and DNA density **[1]**. Except for the peripheral heterochromatin, which showed islands of higher DNA density, the rest of the chromatin looked much more homogeneous and decondensed **[2].**

5.6.1. LAB MEDIA: Figures 5 and 7. *Video editor: Show these figures simultaneously and emphasize figure 7.*

5.6.2. LAB MEDIA: Figure 7.

Pronunciation guide NOTE: ChatGPT gave pronunciation for the words for which pronunciation guides in red are already added in the script. The entire content was run twice but the same response. Hence, the pronunciation guide list is not added here.