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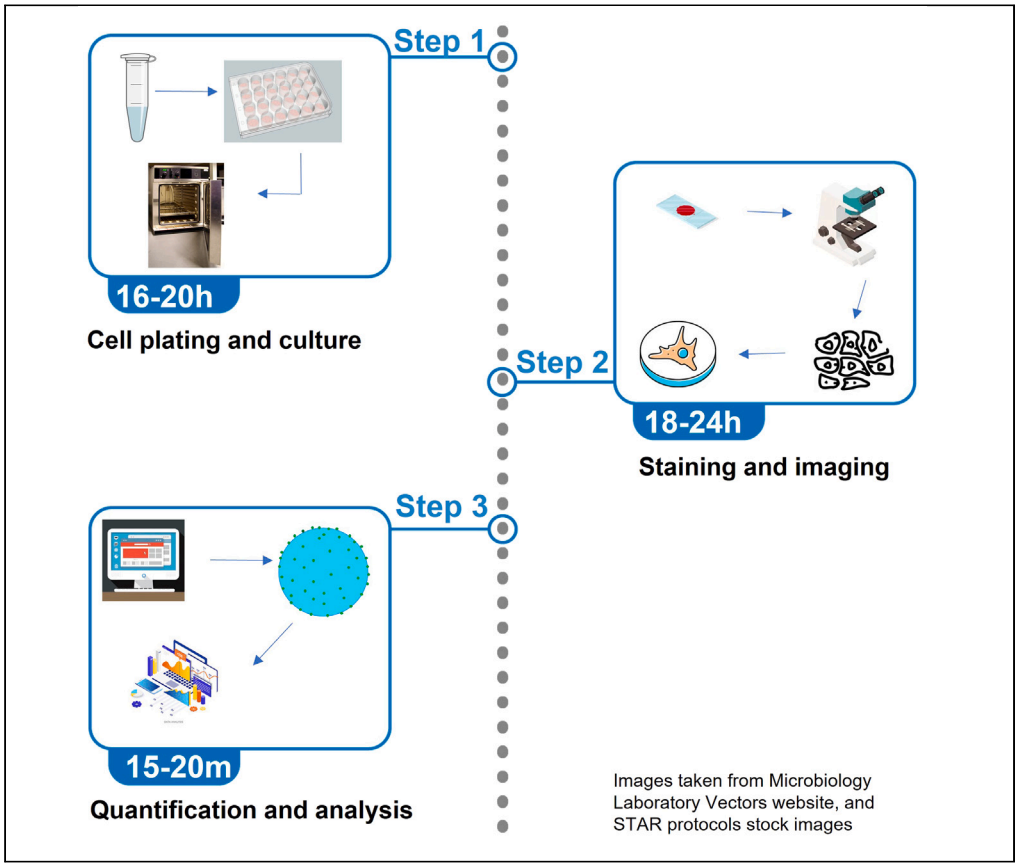
Protocol to image and quantify nuclear pore complexes using high-resolution laser scanning confocal microscopy



Nuclear pore complexes are pathways for nuclear-cytoplasmic communication that participate in chromatin organization. Here, we present a protocol to image and quantify the number of nuclear pore complexes in cells. We describe steps for cell plating and culture, immunofluorescence detection, and confocal microscopy visualization of nuclear pore complexes. We then detail quantification and 3D data analysis. This protocol utilizes digital thresholding under human supervision for quantification of nuclear pore complexes.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Specifications for plating and culturing cells

Immunofluorescence confocal microscopy detection and visualization of nuclear pores

Quantitative image analysis of nuclear pore complex number

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Protocol to image and quantify nuclear pore complexes using high-resolution laser scanning confocal microscopy

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SUMMARY

Nuclear pore complexes are pathways for nuclear-cytoplasmic communication that participate in chromatin organization. Here, we present a protocol to image and quantify the number of nuclear pore complexes in cells. We describe steps for cell plating and culture, immunofluorescence detection, and confocal microscopy visualization of nuclear pore complexes. We then detail quantification and 3D data analysis. This protocol utilizes digital thresholding under human supervision for quantification of nuclear pore complexes.

For complete details on the use and execution of this protocol, please refer to Han et al.[1]

BEFORE YOU BEGIN

Nuclear pore complexes are pathways for nuclear-cytoplasmic communication and participate in chromatin organization.[2] Nuclear pore complexes consist of nucleoporins (Nups), which are arranged in eight-fold symmetry to form a hollow cylinder with approximately ~120–180 nm outer diameter. Widely available approaches for detection and visualization of nuclear pore complexes are needed. We present a protocol utilizing immunofluorescence detection with confocal microscopy visualization of nuclear pore complexes in conjunction with antibody-detection of cytoskeletal structures for co-localization studies. The quantitative results of this protocol were cross-validated with Stochastic Optical Reconstruction Microscopy (STORM) and freeze-fracture replica electron microscopy.[1]

The use of immunofluorescence confocal microscopy[1,3] has advantages over microscopy methods that offer higher resolution. Freeze-fracture electron microscopy,[4] the classical method for nuclear pore complex quantification, requires specialized equipment and skills, and confocal microscopes are more widely available than the equipment for freeze-fracture replica electron microscopy. Likewise, the equipment for super-resolution light microscopy and, especially, for recently introduced lattice light-sheet microscopy[5] to image nuclear pore complexes, is not as available as that for confocal light microscopy. Two, in terms of imaging resolution, freeze-fracture electron microscopy and super-resolution light microscopy can reveal the size of the nuclear pore complexes, but, at this point of development, their resolution cannot reveal the inner molecular structure of nuclear pore complexes. As such, the resolution-advantage over confocal microscopy is small. Three, immunofluorescence confocal microscopy offers to quantify nuclear pore complexes in an entire nucleus. In contrast, freeze-fracture replica electron microscopy exposes only parts of a nucleus, and the entire nucleus cannot be visualized.[4] As such, confocal microscopy eliminates

one possible source of bias, i.e., selection bias. Four, immunofluorescence confocal microscopy offers the combination with multiple antibody markers,[1,3] which enables more sophisticated experiments. Combination with other imaging methods or markers is not possible with freeze-fracture replica electron microscopy because it images the replica of the tissue, i.e., the source tissue, including the epitopes for antibody detection, are not present. Although super-resolution light microscopy can image in different wavelengths, these applications are not as straight-forward as with confocal microscopy. In summary, the combined advantages of the presented confocal microscopy protocol over microscopy approaches with higher resolution outweigh the disadvantages for the visualization and quantification of nuclear pore complexes.

The presented protocol was designed for rat and mouse heart muscle cells (cardiomyocytes), and human induced pluripotent stem cell-derived cardiomyocytes (hiPSCs), and for antibody-based co-localization studies.[1] However, this protocol could be combined with antibody-detection of signaling molecules for nuclear import studies in any cell type.

Cell plating preparation

Timing: 1 h 15 min

1. Prepare 0.025 mg/mL of Human Fibronectin; Dissolve 1 mg of Human Fibronectin in 40 mL of sterile 1× PBS. Pipet up and down gently to thoroughly dissolve. Aliquot and store in −80°C freezer for up to 6 months.
2. Use sterile technique to place a 13 mm Thermanox coverslip into a well of a 24 well cell culture plate.

   Alternatives: An ibidi optical polymer chamber or dish may be used in lieu of coverslips and 24 well plate.

3. Coat the coverslip or ibidi container with approximately 400 µL of sterile Human Fibronectin, enough to cover the entire well. Incubate at 20°–25°C for at least 1 h prior to plating cells.

   Note: This protocol uses treated Thermanox plastic coverslips for cell culture to improve the adherence of cardiomyocytes and hiPSC's. Traditional precleaned glass coverslips should be used for other cell types when appropriate. It is suggested to modify the sample mount for optimal light path properties for each application of this protocol.

Solution preparation for cell fixation and staining

Timing: 15 min

4. Prepare 4% paraformaldehyde Fixation Buffer: 4 mL 10× PBS + 10 mL 16% PFA + 26 mL water.
5. Prepare 0.5% Triton-X100 Permeabilization buffer: 250 µL Triton-X100 + 48.5 mL of 1× PBS.
6. Prepare 3% BSA Blocking solution: 1.5 g BSA (Bovine Serum Albumin) + 48.5 mL 1× PBS or 1× TBS.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Hoechst 33342, Trihydrochloride, Trihydrate – 10 mg/mL solution in water (1:400-1:1000)	Invitrogen	Cat# H3570 Lot#1860907; RRID: N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
NUP Complex Protein Antibody Mab414 (1:400)	Abcam	Cat#; ab24609 Lot# GR-3235621-11 RRID:AB_ 448181
Anti-NUP98 mAb (1:200)	Abcam	Cat#; ab50610 Lot# GR3212026-6[RRID:AB_ 881769
Anti-NUP107 polyclonal antibody (1-2 μg/mL))	Abcam	Cat#; ab73290 Lot# GR6666-28 RRID:AB_1269604
Anti-NUP153 mAB (1:400-1:500)	Abcam	Cat#; ab24700 Lot# GR3177964-2 RRID:AB_2154467
Anti-NUP133 mAb (1:100 for IF 1:100-1:1000 WB)	Santa Cruz	Cat#; sc376763 Lot# E2317 RRID:AB_2889360
Anti-Cardiac Troponin I antibody (1:200- 1:400)	Abcam	Cat# ab56357; Lot# GR313459-8 RRID:AB_880622
Donkey anti-Goat IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (1:200-1:400)	Thermo Fisher	Cat# A21447 Lot# 2045332 RRID: AB_141844
Donkey anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1:200-1:400)	Thermo Fisher	Cat# A21202 Lot# 2018296 RRID: AB_141607
Donkey anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 (1:200-1:400)	Thermo Fisher	Cat# A10037 Lot# 2110843 RRID: AB_2534013
Donkey anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (1:200-1:400)	Thermo Fisher	Cat# A21203 Lot# 1722995 RRID: AB_141633
Donkey anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1:200-1:400)	Thermo Fisher	Cat# A21206 Lot# 2072687 RRID: AB_2535792
Donkey Anti-Rabbit IgG H&L (Alexa Fluor 555) (1:200-1:400)	Abcam	Cat# ab150074 Lot# GR3241278-3 RRID: AB_2636997
Donkey anti-Rat IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1:200-1:400)	Thermo Fisher	Cat# A21208 Lot# 2482958 RRID: AB_2535794
Anti-rat IgG (H + L) Highly Cross-Adsorbed, CF 568 antibody produced in donkey (1:200-1:400)	Sigma	Cat# SAB4600077 Lot# 21C0715 RRID: AB_2827516
Software and algorithms		
Nikon NIS Elements 4.5 software	Nikon Instruments	(https://www.microscope.healthcare.nikon.com/products/software/nis-elements/viewer
Fiji (ImageJ)	Fiji open source software	https://imagej.net/software/fiji/downloads
GraphPad Prism 7	GraphPad Software	N/A
Other		
Electron Microscopy Science Company (EMS) 16% paraformaldehyde	Fisher Scientific	50-980-487
Gibco Antimycotic-Antibiotic 100×	Fisher Scientific	15-240-062
Gibco PBS (1×) pH7.4	Thermo Fisher	10010023
Gibco PBS (10×) pH7.4	Thermo Fisher	70011044
FBS USDA qualified	Fisher Scientific	10437028
DMEM media, high glucose w/o L-glut	Fisher Scientific	11960044
Bovine serum albumin (BSA)	Fisher Scientific	BP9706-100
Glycerol	Sigma	G5516-1L
Triton X-100	Fisher	BP151-500

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Propyl gallate (NPG)	Sigma	02370
Ibidi USA μ-Dish 35 mm, High, ibiTreat - Tissue Culture Treated Polymer Coverslip	Fisher	50-305-806
Corning Centrifuge Tube Top Vacuum Filters 50 mL	Fisher Scientific	Cat. # 09-761-34
Corning Falcon Polystyrene microplates, 24 well	Fisher Scientific	Cat. # 08-772-1
Nunc Thermanox Coverslips, 13 mm	Thermo Fisher Scientific	Cat. # 174950
Colorfrost Precleaned Microscope slides	Fisher Scientific	Cat. # 12-550-43
Corning Cover glass, 22 × 22 mm, thickness #1.5	Fisher Scientific	Cat. # 12-553-454
High Viscosity Cargille Immersion Oil Type B	Fisher Scientific	Cat. # 12-369A
Human fibronectin	Fisher Scientific	Cat# CB40008
Nikon A1R scanning laser confocal microscope (Body: Nikon Ti Eclipse - inverted, Confocal unit: Nikon A1R HD)	Nikon	NA
Piezo motorized stage Mad City Labs 100 μm NanoDrive piezo, 0.2 nm resolution	Nikon	NA
Stage Nikon Ti-S-ER motorized x,y, stage linearly encoded	Nikon	NA
Z direction scanner; Ti Z drive	Nikon	NA
Illumination light ssource: LED Illumination X-Cite 120 Solid State lasers: 405 nm (DAPI) 488 nm (FITC) 561 nm (TRITC) 640 nm (CY5)	Nikon	NA
Laser scanning confocal unit Nikon A1R HD Galvano scanner Laser power of 15 mW at fiber tip	Nikon	NA
Objective lenses 10×: Plan Apo 0.45 NA, 4.0 mm WD 20×: Plan Apo VC 0.75 NA, 1.0 mm WD 40× oil: Plan Fluor 1.30 NA, 0.20 mm WD 60× oil: Plan Apo Lambda 1.40 NA, 0.13 mm WD 100× oil: Plan Apo Lambda 1.45NA, 0.13 mm	Nikon	NA
Wavelength selection Dichroic mirror for excitation and emission	Nikon	NA
Acquisition Software: Nikon Elements version 5.41.02, 64bit	Nikon	NA
Detector (confocal): A1-DUG multi detector with GaASP DAPI: PMT FITC: GaAsP TRITC:GaAsP Cy5: PMT	Nikon	NA
Acquisition mode: Laser scanning	Nikon	NA
Scan Mechanism: Galvano	Nikon	NA

MATERIALS AND EQUIPMENT

All media and solutions used for culturing cells should be prepared using sterile equipment under a biohazard cabinet.

Fibronectin

Reagent	Final Concentration	Amount
Sterile 1× PBS	N/A	40 mL
Human Fibronectin	N/A	1 mg
TOTAL	**N/A**	**40 mL**

Gently pipet up and down until all traces of powder are dissolved. Aliquot and store at −80°C for up to 6 months.

Culture Media		
Reagent	Final Concentration	Amount
Dulbecco's Modified Eagle Medium (DMEM), high glucose w/o L-glut	N/A	44.5 mL
Fetal bovine serum (FBS), USDA qualified	10%	5 mL
Gibco Antibiotic-Antimycotic 100×	1%	500 µL
TOTAL	N/A	50 mL
Sterilize using a 0.22 µm bottle top vacuum filter and store at 4°C for a month.		

Fixation Buffer (4% PFA)		
Reagent	Final Concentration	Amount
EMS 16% paraformaldehyde Aqueous solution	4%	10 mL
PBS 10×	1×	4 mL
MiliQ or deionized water	N/A	26 mL
TOTAL	N/A	40 mL
Store at 4°C for up to 6 months		

Permeabilization Buffer		
Reagent	Final Concentration	Amount
PBS	1×	50 mL
Triton X-100	0.5%	250 µL
TOTAL	N/A	50 mL
Store at 20–25°C for up to 1 year.		

Blocking Buffer		
Reagent	Final Concentration	Amount
PBS	1×	50 mL
Bovine Serum Albumin, DNase and Protease-free	3%	1.5 g
TOTAL	N/A	50 mL
Store at 4°C for up to 6 months.		

Mounting Media		
Reagent	Final Concentration	Amount
Glycerol	N/A	50 mL
N-Propyl gallate (NPG)	1%	0.5 g
TOTAL	N/A	50 mL
Store at 4°C for up to 1 year.		

Note: Rock at 20–25°C at a speed of 2.5–3 rpm and a tilt of 10 for 24–48 hrs. until no visible trace of white powder.

STEP-BY-STEP METHOD DETAILS

Part 1: Cell plating and culture

Timing: 16–24 h

Part 1 of this protocol describes optimal seeding and culturing of cells to ensure proper distribution for clear imaging.

1. Remove fibronectin from each well containing a Thermanox plastic coverslip (or ibidi optical polymer dish or chamber) using a p1000 pipette or aspiration system.
2. Plate an appropriate number of cells per coverslip to achieve 80–90% confluency at time of fixation. Add approximately 400–500 μL of culture media to each well, so that the cells are completely covered to prevent drying out.

   Note: The number of cells needed to achieve the proper confluency is dependent upon cell line and length of cell culture

   ⚠ CRITICAL: Make certain to plate cells at a density that will not cause overcrowding. Cells should be a monolayer at time of fixation.

3. Place the plate in a 37°C 5% CO_2 incubator and incubate 16–24 h.
4. The following morning, remove media and add approximately 500 μL of sterile 1× PBS to each well.
5. Gently rinse the cells by running PBS over the coverslip to remove any dead or unattached cells.

   ⚠ CRITICAL: Take care not to disturb attached cells.

6. Remove PBS and add 500 μL of culture media. Continue to incubate cells until ready for fixation.

   Note: If cells need to be treated, after PBS wash, add appropriate culture media with reagents. Continue to incubate cells in the incubator until intended length of cell treatment is concluded.

Part 2: Staining

Timing: 18–24 h

Part 2 of this protocol describes the steps for fixing and staining cells and nuclei for nuclear pore quantification.

7. Remove culture media from cells and wash with 1× PBS. Fix cells in ~500 μL 4% paraformaldehyde for 20 min at 20–25°C.
8. Wash cells 3 times with 1× PBS.

   Note: Fixation time can be optimized for each user with other cell types for best results.

   Alternatives: Cells can be fixed in 100% methanol (MeOH) at −20°C for 10 min followed by washing 3 times with 1× PBS. However, it is recommended that fixations be optimized for each cell type and antibody since not all antibodies will work with MeOH.

   Note: Cells can be store for several months in PBS at 4°C. However, for best results it is recommended they be stained within 4 weeks.

9. Remove PBS from previously fixed cells and permeabilize with ~500 μL of 0.5% Triton-X 100 to completely cover the cells. Incubate for 10 min at 20–25°C.
10. Wash cells one time with 1× PBS and block by adding ~500 μL blocking buffer. Incubate for 2 h at 20–25°C.
11. While blocking, prepare primary antibody master mix.
    a. Dilute primary antibody in blocking buffer using manufacturers recommended dilution.
    b. Calculate a minimum of 400–500 μL per well. If using more than one primary antibody, dilute all antibodies in the same buffer solution to intended concentration.

c. Store at 4°C until ready to use.

12. After 2 h, remove blocking buffer and add diluted primary antibody mixture to each well. Incubate at 4°C for 16–24 h.

Alternatives: Add ~ 100 μL of diluted antibody to the coverslip and cover with a small piece of parafilm to prevent evaporation and drying.

Note: Mab414 (1:400 dilution) (Abcam, ab24609) is used in this example to stain nuclear pores. Many research papers have used monoclonal antibody (Mab414) for detection of nuclear pore complexes. This antibody recognizes the nucleoporins, Nup62, Nup153, and Nup358. Because the Mab414 antibody is commonly used in published studies, we have utilized it in this protocol. However, we have achieved acceptable confocal microscopy images also with a monoclonal Nup98 antibody.

13. The following morning, remove primary antibody and wash cells in 1× PBS and rock for 5 min at 20–25°C. Repeat wash for a total of 3 times.
14. While washing cells, dilute secondary antibody in 1× PBS according to manufacturer's recommended dilution.

Note: If using more than one secondary antibody, dilute all antibodies in the same 1× PBS buffer solution as done in step 11.

15. Remove third PBS wash and add 400–500 μL of secondary antibody solution to each well. Incubate the cells at 20–25°C in the dark for 2 h.

Alternatives: Add ~ 100 μL of diluted antibody to the coverslip and cover with a small piece of parafilm to prevent evaporation and drying.

⚠ CRITICAL: Be sure plate is stored in complete darkness, either in a drawer or covered with aluminum foil.

16. After 2 h, remove secondary antibody and wash cells 3 times in 1× PBS for 5 min as in step 13.
17. Dilute Hoechst in 1× PBS at approximately 1:500–1:1000 or optimize according to manufacturer's recommendation.
    a. incubate at 20–25°C in the dark for 5 min.
    b. Wash briefly in 1× PBS.
18. Using fine-tipped forceps, carefully remove coverslip from the well, taking care not to flip the coverslip or scratch cells from surface of the coverslip (Figure 1A).
19. Mount coverslip onto a precleaned microscope slide with cells facing up between two thin strips of pre-cut parafilm (Figure 1B).

Note: Slides may be cleaned with a Kimwipe and 100% Ethanol to remove dust or lint prior to mounting.

Note: The two parafilm strips act as spacers to avoid pressing cells and damaging cell morphology.

The parafilm strips (approx. 2 mm × 15 mm) should be cut and placed on the microscope slide prior to placing coverslip to avoid drying out the cells. Take care to place the strips apart far enough to fit a 13 mm round coverslip, but still within the range of cover by the square coverslip (22 mm wide) that is to be mounted on top.

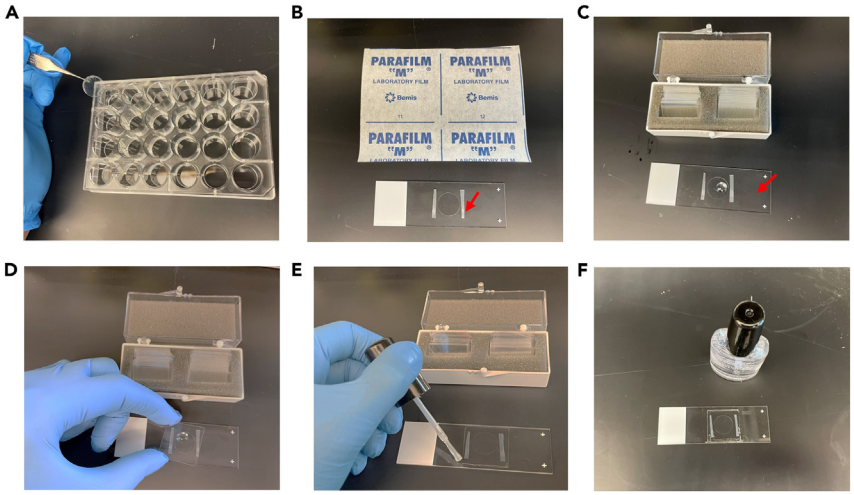

Figure 1. Prepare fixed cells for imaging

(A) Remove coverslip with fixed cells from a 24 well plate.

(B) Coverslip with fixed cells facing up (red arrow) placed on microscope slide with spacers consisting of 2 strips of parafilm used to prevent cells from being flattened.

(C) Add one drop of mounting media (red arrow) to the middle of the round coverslip containing fixed cells.

(D) Cover with a 22 mm × 22 mm glass coverslip #1.5.

(E) Seal all four edges of the square coverslip with nail polish.

(F) Slide is completely sealed with nail polish and ready for imaging.

20. Add 1 drop (approx. 25 μL) of mounting media and cover the cells with 22 mm × 22 mm glass coverslip #1.5, allowing the media to cover the cells while avoiding bubbles (Figures 1C and 1D).
21. Seal the edges of the glass coverslip with fingernail polish. Allow to dry before imaging. (Figures 1E and 1F).

Note: In the presented protocol, special consideration was given to the sample mounting (shown in Figure 2). In the design/development of the sample mounting part of our protocol, we had to consider the following: (1) Optimal attachment of cardiomyocytes requires growth on plastic. To this end, we have seeded cardiomyocytes on round Thermanox coverslips. So as not to distort the light path with the plastic Thermanox coverslips, we have used cover glass for mounting (Figure 2). (2) In our experience, the use of spacers is necessary to limit the compression of nuclei by the coverslips being pulled by capillary forces (Figure 2). (3) For highest possible resolution, we have used an oil-immersion lens.

Part 3: Confocal microscopy

Timing: 45 - 60 m

Part 3 of this protocol describes the procedure for imaging and quantifying nuclear pores using high resolution microscopy.

Note: This protocol utilizes Nikon equipment and software. Confocal microscopes from other manufacturers will enable the same experiments with minimal modifications of this protocol. We provide details of the hardware and terminology used, which should help identify corresponding equipment from other vendors.

22. Whole cell imaging.

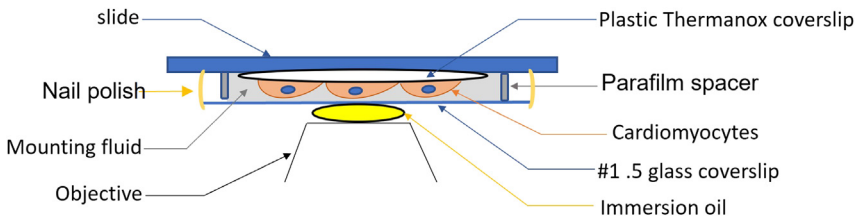

Figure 2. Cross-sectional diagram of sample mount

This protocol for heart muscle cells uses an inverted microscope with a Nikon 100× Plan Apo, Lambda, oil 0.13 mm working distance lens.

a. Place slide containing mounted cells onto the stage of the confocal microscope. The slide should be placed so that the glass coverslip is facing toward the objective. (Figure 2).
b. Using the DAPI channel, locate cell nuclei under the 10× or 20× objective lens.
   i. Switch to the Plan Apo Lambda 100× oil immersion objective lens.
   ii. Add 1 drop of Immersion Oil Type B to the surface of the lens.
   iii. Identify cells with smooth nuclear border by bringing the edge of the nucleus into focus.

⚠ CRITICAL: Only select cells with smooth nuclear border in interphase where the entire nuclear envelope can be seen as a disc. Cells undergoing mitosis should be avoided due to the breakdown of the nuclear envelope.

c. Once a cell has been selected, set the pinhole size to 0.7AU.
   i. Set scanning format size to 1024 × 1024 pixel with a pixel dwell time of 1.1 µsec.
   ii. Press the *SCAN* button to begin scanning.

Note: See Image Acquisition Parameters (Table 1) for suggested settings.

d. Adjust the laser power and gain at the optimal focal plane for each subcellular structure (nucleus, nuclear pores, etc). Modify the focus as needed.

⚠ CRITICAL: Avoid oversaturation (Figure 4A).

Note: Laser power and gain settings for each channel may vary on different systems and with individual users. The overall principal is to avoid over oversaturation.

Note: Because it is important to use the full dynamic range of the detector, it is recommended to evaluate the Look Up Table (LUT) histogram for appropriate display of low through high range of intensity (Figure 4B).

e. Take an image of the cell of interest by selecting *CAPTURE*. The captured image will appear with all fluorophores overlayed. Save both the ND file, the captured raw data containing separate channel images for each fluorophore, and a TIFF file representing all fluorophores (Figure 3).

Note: Terminology or directions for collecting images may vary on different systems.

23. Nucleus and Nuclear Pore Imaging.
   a. Select *SCAN* again and crop the image so the nucleus engulfs the entire scanning field while leaving room to visualize the entire nuclear envelope and surrounding nuclear pores (Figures 4 and 5).

Note: Use the crop function rather than optical zoom to avoid changing the pixel size

Table 1. Image acquisition parameters

Acquisition setup	Settings for Whole cell Imaging	Settings for imaging Nucleus
Acquisition Mode	Laser scanning confocal	Laser scanning confocal
Scan Mechanism	Galvano	Galavano
Line Averaging	0	0
Pinhole	0.7AU	0.4AU
405 nm (DAPI)	Emission 450	Emission 450/50 nm
Laser Power	3%	3%
GAIN	96	106
OFFSET	0	0
488 nm (FITC)	Emission 525	Emission 525/50 nm
Laser Power	1%	1%
GAIN	37	47
OFFSET	0	0
640 nm (CY5)	Emission 700	Emission 685/70 nm
Laser Power	3%	3%
GAIN	90	98
OFFSET	0	0
Pixels	1024 × 1024	2048 × 2048
Pixel dwell time	1.1 µsec	3.2 µsec
Pixel size	0.08 µm/px	0.04 mm/pixel
Image Size	1024 × 1024	368 × 368
Z-stack step size	NA	0.3 mm

b. Adjust the pinhole size to 0.4 AU to decrease the amount of scattered light and increase the resolution to enhance the definition of the nuclear pores.
   i. Set the *scanning* format size to 2048 × 2048 pixel with a pixel dwell time of 3.2 µsec.

Note: See Image Acquisition Parameters (Table 1) for suggested settings.

Note: After decreasing the pinhole size, re-adjust the laser power and gain as needed for each laser to increase the brightness of the subcellular structures. Once the changes have been made, maintain the parameters for all similar acquisitions.

⚠ CRITICAL: Avoid oversaturation (Figure 4).

c. Once the pinhole has been adjusted to 0.4AU and the laser power and gain have been established, obtain a 3D image using the Z-series Acquisition on Nikon Elements software.
   i. Select the *Acquisition* tab and click the *Z-stack* box.
   ii. Set the Z range by setting the top and bottom positions of the nucleus.
   iii. Focus on the top of the nucleus and press the *Top* button.
   iv. Move the focus to the bottom of the nucleus and press the *Bottom* button.
   v. Set the Z-step size to 0.3 µm to cover the entire depth of the nucleus.
   vi. Select *Run Now*. Save image as an ND file. (Figure 5)

Note: Terminology or directions for collecting images may vary on different systems.

Note: In this protocol, we have used a z-step size of 0.3.µm. Using the formula for z-axis resolution of a confocal light path : $r_z = \frac{1.4n\lambda}{NA^2}$ for determining the optical resolution limit in z-axis, we calculated that the combined refractive index of cover glass and glycerol-based mounting medium of approximately 1.45, an emission peak wavelength of 525 nm for Alexa 488, and a numerical aperture (NA) of the objective lens of 1.45 yields a z-axis resolution of 507 nm. As such, this protocol oversamples in z-axis. Users who are interested in reducing microscope time for acquisition could use a wider step size in z-axis as appropriate for their equipment.

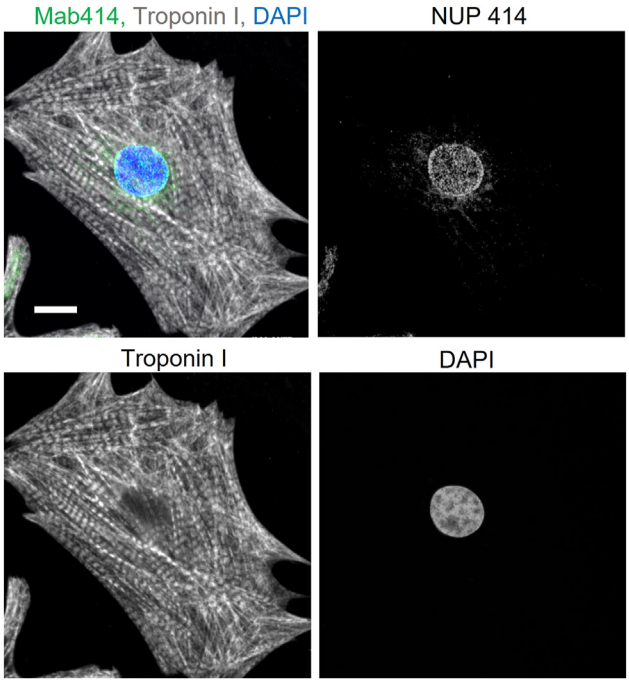

Figure 3. Neonatal rat cardiomyocyte imaged on high resolution with a Plan Apo Lambda 100× oil lens objective using Nikon A1R scanning laser confocal microscope
Scale bar 10 μm.

24. 3D data analysis using Nikon Elements software.

Note: This protocol uses Nikon Elements software to quantify nuclear pore complexes. However, several open-source software options are available which can be used. Steps using Fiji (ImageJ) are outlined in Step 25.

a. Define Binary Threshold.
   i. Convert 3D Z-stack ND file to a Max (maximum) Intensity Projection image (Figures 6A and 6B).
   ii. Right click in the Max Intensity Projection image and select *New Document* (Figures 6C and 6D).
   iii. Using the *New Document* image (Figure 6D), obtain binary threshold under the DAPI channel. Click within the nucleus in several areas. Be sure to select both light and dark regions (Figures 6E and 6G).
   iv. Define parameters in thresholding dialogue box to determine binary (Figures 6F,6H, and 6I).
b. Convert Binary to ROI.
   i. Select the *ROI* tab located on the tool bar above the image (Figure 7A).
   ii. Select *Move Binary to ROI* (Figure 7A).
   iii. Right click within the ROI. Select *Copy ROI* (Figure 7B).
   iv. Paste ROI into the **ORIGINAL** Z-Stack image (Figures 7C and 7D).

⚠ CRITICAL: Do not paste ROI in Max Intensity Projection image. Max Intensity Projection image will not allow for scrolling through the optical sections taken in the Z-stack in step 23c.

c. Select proper Z-stack optical sections for quantification.
   i. In the Z-Stack image, choose the top and bottom sections of the nucleus showing pores on the surface and along the edge of the nuclear envelope (NE) (Figures 9A and 9B).

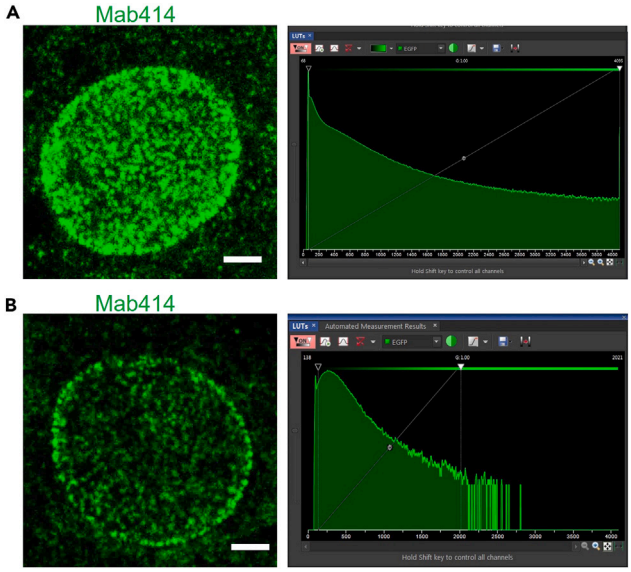

Figure 4. Examples of oversaturation

(A) Oversaturated single plane photomicrograph of nuclear pores. Look Up Table (LUT) histogram showing saturation exceeding 80–90% of pixel gray value. Scale bar 2 μm.

(B) Appropriately saturated single plane photomicrograph of nuclear pores. Look Up Table (LUT) histogram showing saturation within 80–90% of pixel gray value. Scale bar 2 μm.

⚠ CRITICAL: Do not select optical sections that show only the edge of the NE or those that exclude the edge of the NE (Figures 8A and 8B).

ii. Under the *Binary* tab located on the tool bar, select *Spot Detection* -> *Bright Spots* -> *Bright and Clustered* (Figure 9C).
iii. Select the channel used to stain the pores and set parameters to identify nuclear pores. (Figure 9E).
iv. 1px.
v. Diameter 0.19 μm.
vi. Contrast ~17.
vii. Object symmetry = all.
viii. Check Remove dark objects.
ix. Check Apply to all frames.
x. Check Preview.
xi. Select either the top or bottom section of the nucleus to begin the quantification. (Figures 9A and 9B).
xi. Adjust remove *dark objects* in the *spot detection* dialogue box iteratively until there is an appropriate optimum of included and excluded objects (Figure 9E).

d. Quantification of Nuclear Pores.
i. Once the parameters have been defined in the spot detection dialogue box, hit *OK* (Figures 9D and 9E).
ii. Open *Automated Measurement Result* under the *Analysis* tab in the tool bar.
iii. Select ROI DATA in the first drop down box, indicated by a yellow arrow in Figure 10C.
iv. Select ROI AREA in the second drop down box, indicated by a white arrow in Figure 10C.
v. Hit the refresh button, indicated by the top red arrow in Figure 10C. This will give the number of nuclear pores along with the ROI area for that section.
vi. Export data to Excel. Data will include the number of pores as well as the ROI area.

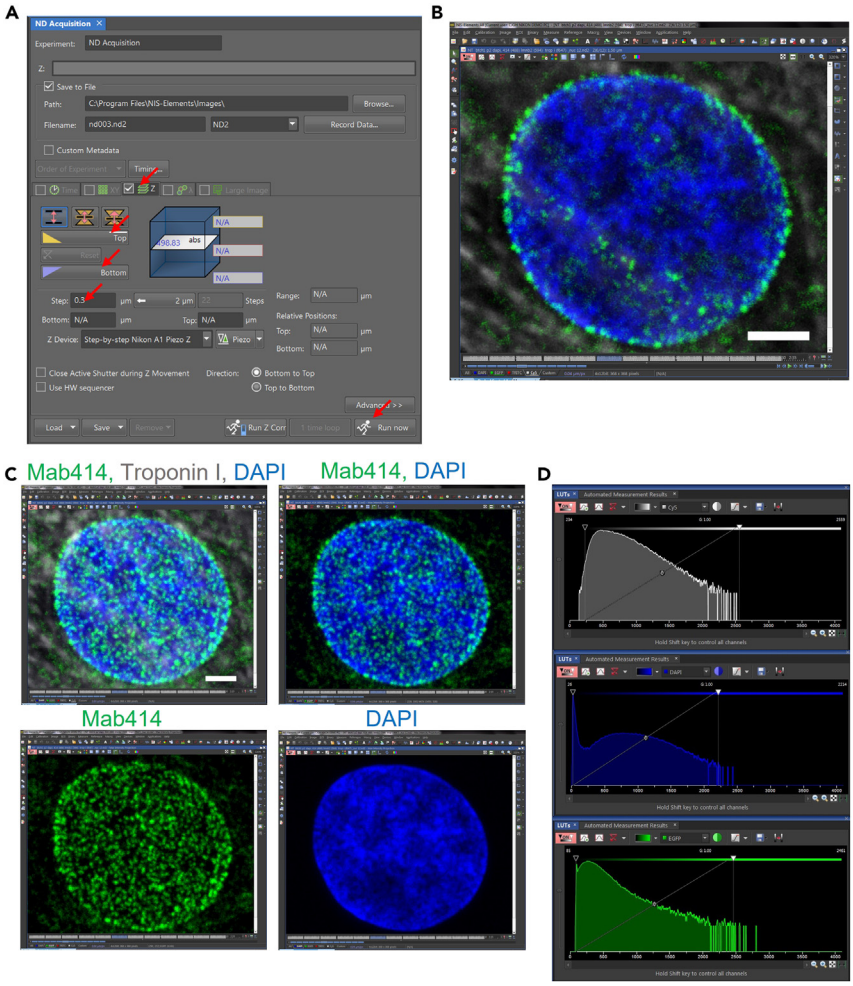

Figure 5. Acquiring Z-stack image Next step of appropriately saturated nucleus in Figure 4B

(A) Screen shot of the *ND Acquisition* window to demonstrate setting z-stack parameters.

(B) Z-stack ND file image of neonatal rat cardiomyocyte acquired using Plan Apo Lambda 100× oil 1.45NA, 0.133 mm lens objective. Scale bar 2 μm.

(C) 2D Max projection image of scanned nucleus showing merged image along with separate channels. Scale bar 2 μm.

(D) Look Up Table (LUT) indicating appropriate saturation levels.

vii. Without changing any settings, scroll down to the other optical section (top or bottom) and hit the refresh button again (Figure 10C). The number of pores will be updated.

viii. Export data.

ix. To calculate the total number of pores for the nucleus, add the two numbers. For area density, divide the total number of pores by the area of the ROI (nucleus) (Figure 10C).

25. 3D data analysis using Fiji (image J).

Note: Here we outline steps to quantify nuclear pores using Fiji (Image J) with and without the utilization of Gaussian Blur to reduce image noise:

a. Quantification steps using Gaussian Blur (Figures 11A–11E):

i. Load raw data file into ImageJ.

ii. Create 2 duplicates of the of the original file for the channel used to detect nuclear pores and name as 1 and 2.

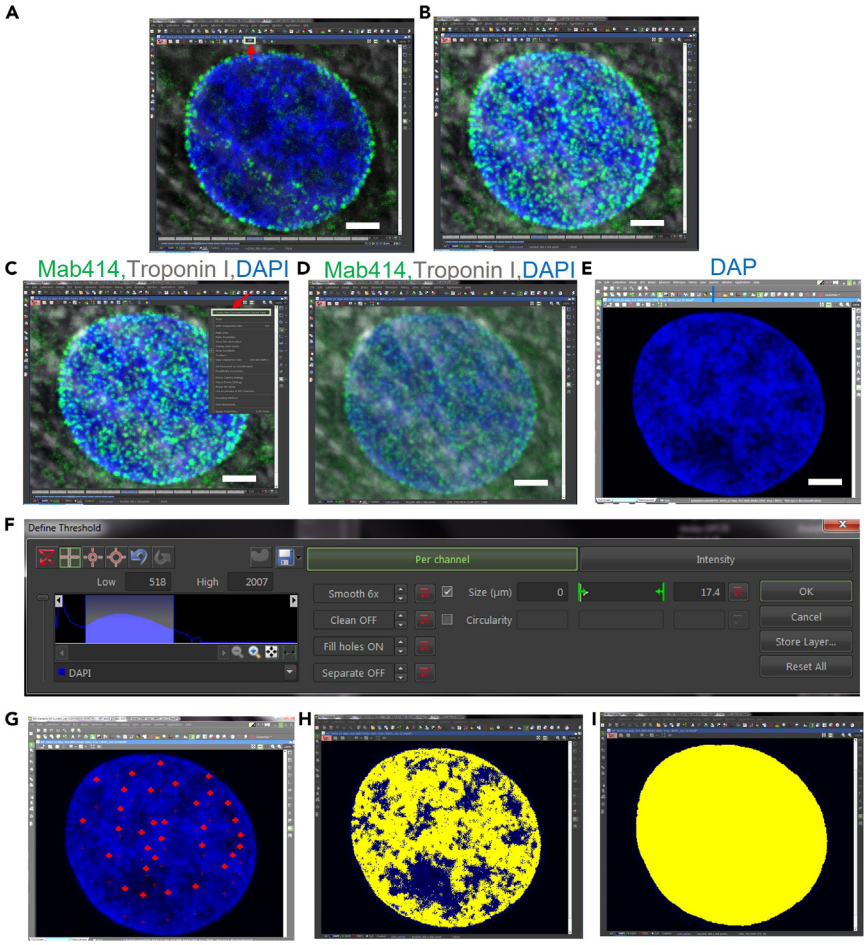

Figure 6. Obtain max projection and define binary

(A) Z-stack image of nucleus. Red arrow indicates tab used to obtain a max projection image Scale bar 2 µm.

(B) Max projection image of nucleus. Scale bar 2 µm.

(C) Max projection image of nucleus. Red arrow indicates drop down box used to "create new document" to define binary threshold. Scale bar 2 µm.

(D) New document used to define threshold Scale bar 2 µm.

(E) Select DAPI Channel under New Document used to define binary.

(F) Screen shot of the dialogue box for defining binary threshold parameters.

(G–I) (G) Select area of contrast to define binary (H) Thresholding after binary threshold is defined (I) Final Threshold after additional parameters are adjusted, including *Smooth, Clean, Fill holes, Separate.*

iii. Threshold on the DAPI channel to create an ROI of the nucleus.
iv. Create ROI: Edit -> selection ->create selection.
v. Copy ROI to both duplicates.
vi. Create a Gaussian Blur on each of the duplicates. Process ->Filter -> Gaussian Blur.
vii. For Image 2 use a Sigma (radius) of 2. For Image 1 use a Sigma (radius) of 1.
viii. Find the Difference of Gaussians (DoG) by subtracting image 2 from image 1.
ix. Process -> Image Calculator.
x. Choose "1" as image 1 and "2" as image 2.
xi. Operation = subtract.
xii. Create new window.
xiii. Adjust brightness and contrast.
xiv. Threshold the nuclear pores on the DoG image.

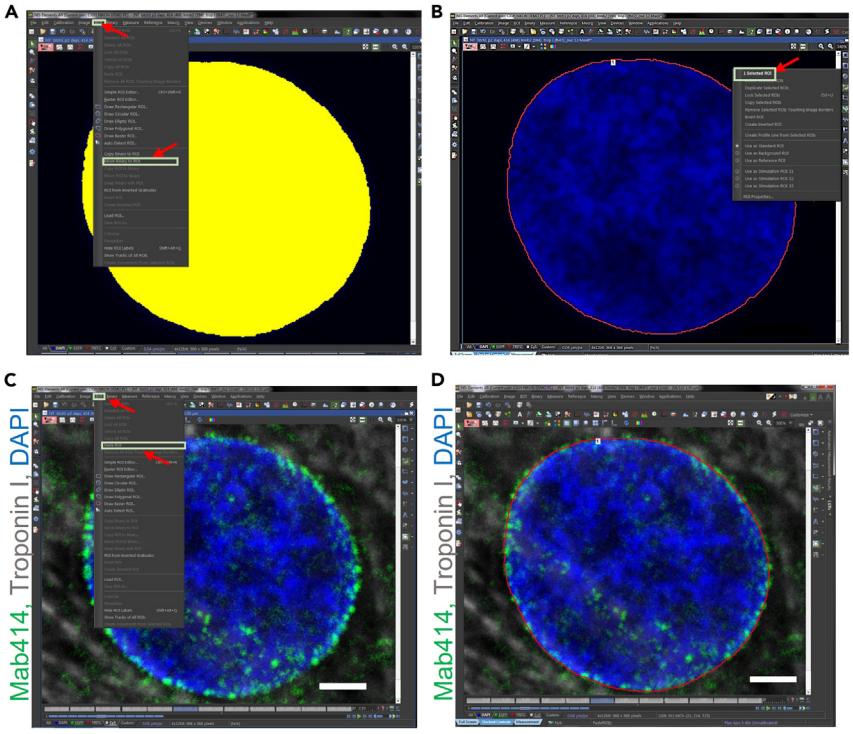

Figure 7. Convert Binary to ROI

(A) Image of final binary threshold. Red arrow indicates the dropdown box used to *Move binary to ROI*.

(B) Image of binary converted to ROI. Red arrow indicates selection of ROI in dropdown box.

(C) Original Z-stack image. Red arrow indicates the dropdown box used to *Paste ROI* from thresholding.

(D) Original Z-stack image showing ROI which will be used for nuclear pore quantification. Scale bar 2 μm.

xv. Separate particles: Process -> Binary ->Watershed.

xvi. If needed, remove errant particles. Process -> Binary -> Open.

xvii. Set size measurements. Analyze ->Set measurement.
Check Limit Threshold box.
Check Bounding rectangle box to count particles within the ROI only.

xviii. Count Pores.

xix. Analyze ->Analyze particles.
Set size parameters and additional outputs needed.
Hit OK. Summary will show total number as well as other overall measurements.

b. Quantification Steps without the use of Gaussian Blur (Figures 11F–11I):

i. Load raw data file into image J.

ii. Threshold on the DAPI channel to create an ROI of the nucleus.

iii. Binary -> open.

iv. Edit -> selection -> create selection for ROI.

v. Copy ROI.

vi. Adjust brightness and contrast.

vii. Threshold the nuclear pores.

viii. Separate particles: Process -> Binary -> Watershed.

ix. Set size measurement: Analyze -> Set measurement.

x. Check Limit Threshold box.

xi. Check Bounding rectangle box to count particles within the ROI only.

xii. Count pores.

xiii. Analyze -> Analyze particles.

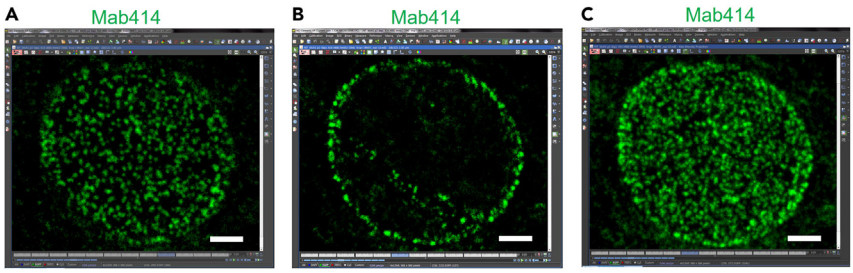

Figure 8. Identify correct Z-stack optical sections for counting nuclear pores

(A) Optical section in Z-stack showing nuclear pores only on the surface of the nuclear envelope. Scale bar 2 μm.

(B) Optical section in Z-stack showing nuclear pores only along the edge of the nuclear envelope. Scale bar 2 μm.

(C) Optical section in Z-stack showing nuclear pores along the edge and surface of the nuclear envelope. Scale bar 2 μm.

Set size parameters and additional outputs needed.

Hit ok. Summary will show total number as well as other overall measurements.

EXPECTED OUTCOMES

This protocol could be used on different cell types for detecting and quantifying nuclear pores along the nuclear envelope. This protocol shows that cardiomyocyte nuclei have 1,856 ± 66 in fetal (E14.5), 1,040 ± 53 in neonatal (P2), and 678 ± 32 in adult (P60) rat hearts (mean ± SEM, ref. 1. However, the number of nuclear pore complexes varies widely between cell types and growth conditions,[6,7] and, consequently, the quantification may yield different results in other cell types.

LIMITATIONS

This protocol outlines a reproducible approach for quantifying pores in the nuclear envelope. The pores are quantified by obtaining 3D z-stack images and compressing them into 2D maximal intensity images. This allows for the pores to be quantified along the nuclear surface and edge within the same optical section. However, this may cause the software to pick-up small background signals and include them as part of the quantification. In addition, nuclear pore complexes are made up of approximately 30 different nucleoporins which gives it the cylindrical structure of eight spokes surrounding a central tube. Since this protocol is used to detect only certain nucleoporins, the structure or the transport activity of the pore cannot be determined. It should be emphasized that this protocol assumes that nuclei have the shape of a flat cylinder and quantifies the nuclear pore complexes of the parallel surfaces while neglecting the mantle surface. This should be considered when this protocol is used for nuclei with a round or irregular shape. Moreover, this protocol relies on the quality of the antibodies to detect nuclear pores. Nonspecific binding or weak binding could yield false positive or false negative counts of the nuclear pores, respectively. It is, however, worth noting that the quantitative results of nuclear pore numbers in cardiomyocytes using this protocol and antibody (Mab414), were cross validated with Stochastic Optical Reconstruction Microscopy (STORM) and freeze fracture replica electron microscopy (EM), as published.[1] Applications in other cell types should consider similar cross validations.

TROUBLESHOOTING

Problem 1

Low or no signal (related to steps 22 and 23; Figure 12).

Potential solution

- Be sure that cells have not been kept in storage for a long period of time prior to staining.
- Use newly purchased antibody and aliquot to avoid freeze/thaw cycles. Using an antibody that is old or has been through multiple freeze/thaw cycles will decrease the signal.
- Use correct antibody dilution. Follow vendor's recommendation and increase concentration if the age and status of the antibody is unknown (for example, From 1:400 to 1:200).

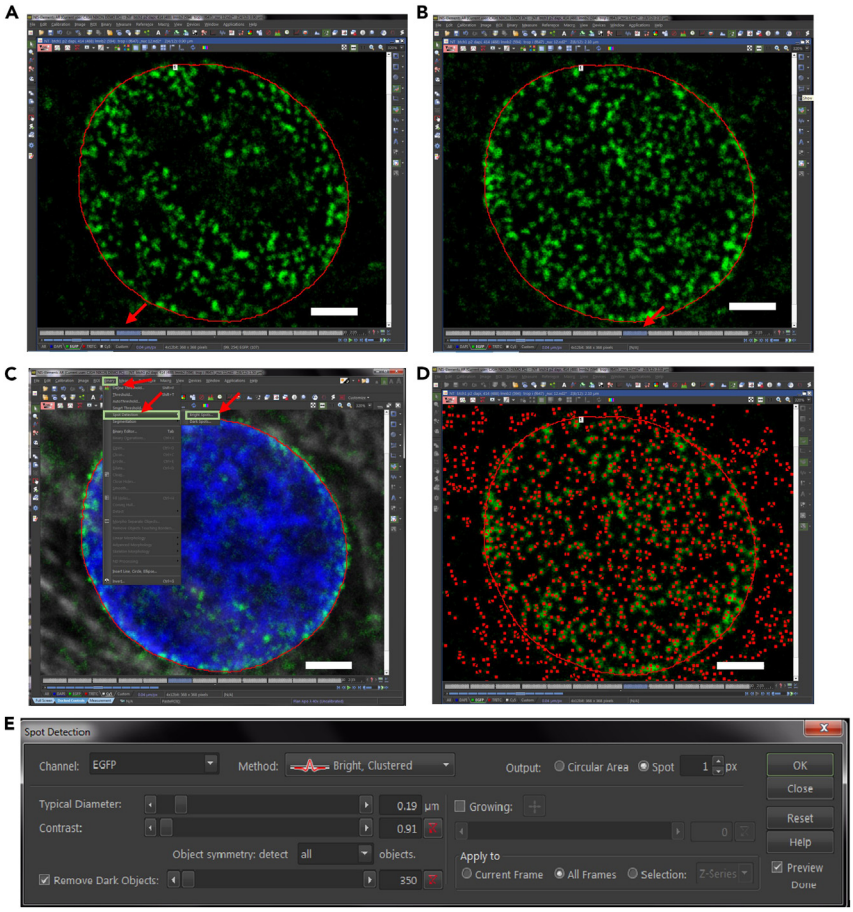

Figure 9. Identify nuclear pores stained with Mab414 using spot detection with Nikon Elements Software

(A) Lowest optical section in Z-stack, indicated by red arrow, used for counting nuclear pores along the edge and surface of the nuclear envelope. Scale bar 2 µm.

(B) Highest optical section in Z-stack, indicated by red arrow, used for counting nuclear pores along the edge and surface of the nuclear envelope. Scale bar 2 µm.

(C) Original z-stack image. Red arrows indicate *spot detection* and *bright spots* located under the *Binary* tab. Scale bar 2 µm.

(D) Nuclear pores detected by spot detection prior to parameters adjustment. Scale bar 2 µm.

(E) Zoomed-in screen shot of *Spot Detection* box used to set parameters for selecting nuclear pores to be counted.

- Be sure that incubation of the secondary antibody is done in complete darkness.
- Check to ensure that the secondary antibody used matches the host of the primary antibody.
- Image cells within a day or two of staining to prevent the signal from fading.

Potential problem 2

High background (related to step 22 and 23; Figure 13).

Potential solution

- Rocking the plate during each wash step will ensure removal of any excess fixative, primary and secondary antibody solutions as well as any loosely bound antibody.
- Be sure to use fresh fixative. Old or expired formaldehyde may cause autofluorescence.
- Change secondary antibody to one that is known to have less background (i.e., Alexa Fluor 594).
- Decrease pinhole to reduce amount of scattered light.
- Decrease density of cells plated to prevent overlapping.

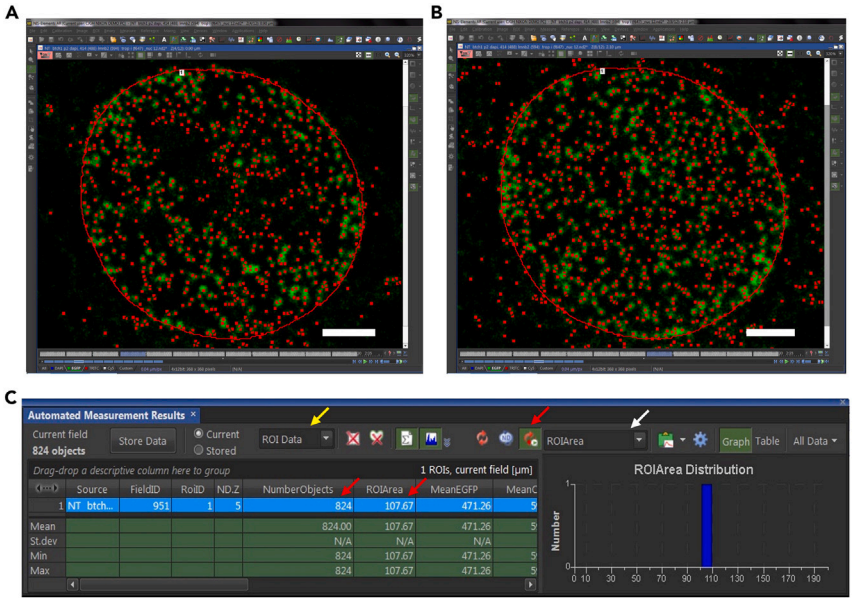

Figure 10. Quantification of nuclear pores stained with Mab414

(A) Nuclear pore quantification on the lowest optical section in the Z-stack showing pores along the edge and surface of the nuclear envelope. Scale bar 2 µm.

(B) Nuclear pore quantification on the highest optical section in the Z-stack showing pores along the edge and surface of the nuclear envelope. Scale bar 2 µm.

(C) Screen shot image of *Automated Measurement Results* dialogue box. Yellow arrow indicates "ROI Data" drop down box; white arrow, "ROI Area" drop down box; red arrows, Number of Objects (nuclear pore count), ROI Area, and refresh button used to obtain counts from both sections.

Problem 3

Over selection of bright spots by software (related to step 24c; Figure 14).

Potential solution

Adjust parameters in spot detection dialogue box, i.e., *contrast* and *remove dark spot*.

Problem 4

Decreased or non-attachment of cells (related to Part 1).

Potential solution

- Check for bacterial contamination.
- Check preparation and coating conditions of Fibronectin
- Test different extra cellular matrix for coating coverslips prior to plating (for example: Collagen, Laminin, or Gelatin). Other cell types may require a different matrix. Prepare and coat according to manufacturer's recommendations.

Problem 5

During imaging, cells do not come into focus or remain in focus (related to step 22 a and b).

Potential solution

- Check to confirm that the coverslip was not flipped during mounting so that the cells are facing the slide and not the No. 1.5 cover glass (steps 18 and 19)

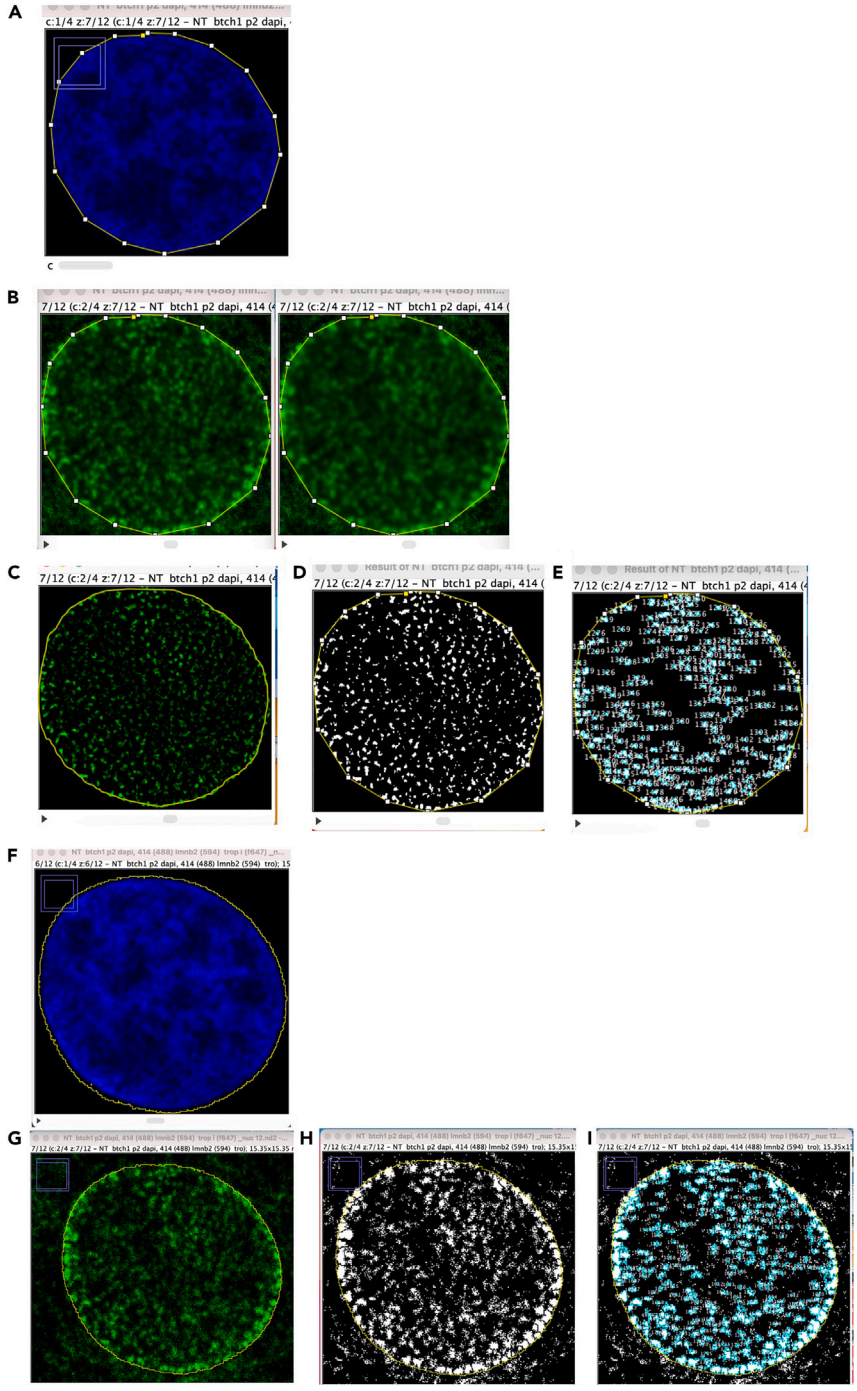

Figure 11. Quantification of nuclear pores stained with Mab414 using Fiji/ImageJ

(A–I) Workflow for quantification with (A–E) and without (F–I) Gaussian blurring. (A) DNA staining with Hoechst to delineate the boundaries of the nucleus. (B) Use of Gaussian Blur on photomicrograph of nuclear pores stained with Mab414 with sigma radius of 1 (left panel) and 2 (right panel). (C) Difference of Gaussians (DoG): Subtraction of photomicrograph 2 (sigma 2) from photomicrograph 1 (sigma 1). (D) Thresholding and binary on nuclear pore complex image. (E) Digital quantification yields nuclear pore count of 261. (F) DNA staining with Hoechst to delineate the boundaries of the nucleus. (G) Photomicrograph of nuclear pores stained with Mab414. (H) Thresholding and binary on nuclear pore complex image. (I) Digital quantification yields nuclear pore count of 369.

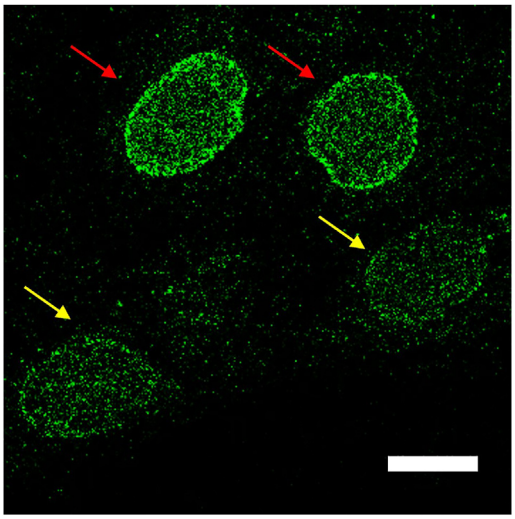

Figure 12. High resolution image of nuclear pores stained with Mab NUP 153

Induced pluripotent stem cell cardiomyocytes (ips-cm's) acquired using Plan Apo Lambda 100× oil 1.45NA, 0.13 mm objective lens. Red arrows indicate high nuclear pore antibody signal. Yellow arrows indicate low nuclear pore antibody signal. Scale bar 10 μm.

- Checked to be sure that the No. 1.5 glass coverslip is sitting evenly on top of the parafilm spacers and plastic Thermanox coverslip (steps 20 and 21)
- Check that enough oil has been placed on the objective so that it spreads evenly once the objective has come into contact with the glass coverslip,

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bernhard Kuhn: bernhard.kuhn2@chp.edu.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any data sets or codes.

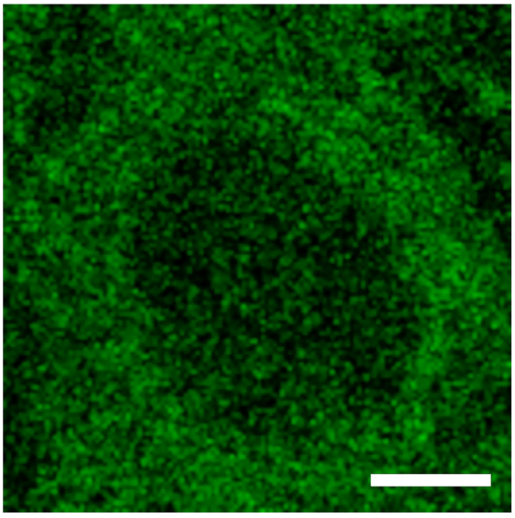

Figure 13. Zoomed in high resolution image of nuclear pores stained with Mab NUP 153

Mouse cardiomyocyte nucleus acquired using Plan Apo Lambda 100× oil 1.45NA, 0.13 mm objective lens showing high background staining. Scale bar 5 μm.

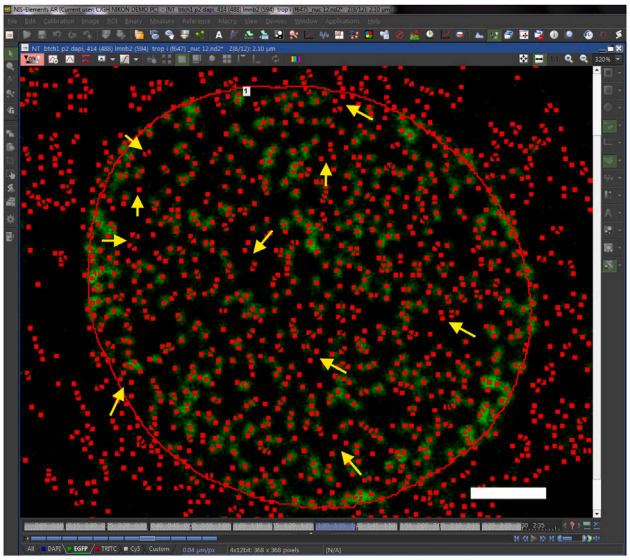

Figure 14. Over counting of nuclear pores by Elements software
Yellow arrows indicating several areas over counted by Elements. Scale bar 2 μm.

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AUTHOR CONTRIBUTIONS

J.D.M.-B.: performed experiments, data analysis, and manuscript writing. B.K.: conceived research approach, study design and manuscript writing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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