Chapter 6

Visualization of the Nucleolus in Living Cells with Cell-Penetrating Fluorescent Peptides

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Abstract

The nucleolus is the hallmark of nuclear compartmentalization and has been shown to exert multiple roles in cellular metabolism besides its main function as the place of ribosomal RNA synthesis and assembly of ribosomes. The nucleolus plays also a major role in nuclear organization as the largest compartment within the nucleus. The prominent structure of the nucleolus can be detected using contrast light microscopy providing an approximate localization of the nucleolus, but this approach does not allow to determine accurately the three-dimensional structure of the nucleolus in cells and tissues. Immunofluorescence staining with antibodies specific to nucleolar proteins albeit very useful is time consuming, normally antibodies recognize their epitopes only within a small range of species and is applicable only in fixed cells. Here, we present a simple method to selectively and accurately label this ubiquitous subnuclear compartment in living cells of a large range of species using a fluorescently labeled cell-penetrating peptide.

Key words Cell permeable nucleolar marker, Cell-penetrating peptide, Fluorescence microscopy, Living cells, Nucleolus, Poly-arginine

1 Introduction

In 1831, a Scottish botanist Robert Brown described for the first time the "nuclei" in plant cells [1]. A few years later, in 1836, Gabriel Gustav Valentin working with neuronal cells described within nuclei a prominent substructure later termed "nucleolus" [2]. It was only one century later, in the 1960s, that findings derived from electron microscopic autoradiography of [^{3}H]uridine incorporation established the major function of the nucleolus in rRNA synthesis, rRNA processing, and ribosome biogenesis [3, 4]. Several other tasks have been more recently added to the functional portfolio of the nucleolus (reviewed in [5, 6]).

The nucleolus is a self-organizing structure not delimited by membranes that gets disassembled and reassembled around the rDNA loci at every cell division cycle ([7]; reviewed in [8]).

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Transcription of ribosomal genes, which make up the fibrillar centers, takes place surrounding this structure and creates the so-called dense fibrillar component [9, 10]. Further processing of the precursor RNA and multiple proteins gives rise to ribosome precursor particles, which form the granular component (reviewed in [11]). Over 300 nucleolar proteins have been identified by proteomics [12]. The first nucleolar targeting sequences were identified in viral proteins ([13, 14]; reviewed in [15]) and further shown to be able to cross cellular membranes [16, 17]. Recently, we performed a systematic analysis of peptide sequences sufficient for nucleolar targeting [18]. We found that six or more arginines are sufficient to label the nucleolus by binding to RNA. Hence, these peptide sequences can label the nucleolus in all species tested (insect, fish, mouse, human, and very likely also yeast). Importantly, in particular nona- and deca-arginine peptides are similarly highly active in cell membrane permeation [19].

In this chapter, we describe how adding these peptides conjugated to a fluorescent label directly to the cell culture medium without the need for transient or stable transfections of the cells provides a live cell fluorescent nucleolar marker in a variety of species [20]. The nucleolar marker is membrane permeable and reaches its target within minutes. To prevent proteolytic degradation the peptide can be synthetized with D-amino acids. The cell-penetrating-peptide-based marker provides a method for the noninvasive marking of the nucleolus in living cells, allowing the three-dimensional reconstruction of the nucleolus, which is not possible with contrast microscopy techniques. Besides that, it can easily be used in combination with different fluorophores like fluorescent proteins or DNA dyes to correlate different aspects of nuclear structures with the nucleolus. In addition, nucleolar labeling is also preserved during fixation and staining of the cells. Furthermore, it does not affect cellular viability, proliferation, and rDNA transcription.

2 Materials

Prepare all solutions at room temperature unless otherwise noted. Solutions used to culture and prepare cells should be warmed to 37 °C prior to use. All solutions and materials in contact with live cells must be sterile and have to be handled inside a tissue culture biosafety cabinet. Disposal of reagents, solutions, and cell material has to be carried out following the specific regulations in effect.

2.1 Cells and Materials for Cell Culture

1. Human HeLa cervix epithelium adenocarcinoma cells.
2. Adult rat ventricular cardiomyocytes.
3. Mouse C2C12 myoblast cells.
4. Pac2 zebrafish fibroblast cells (*Danio rerio*).
5. Sf9 insect ovary cells (*Spodoptera frugiperda*).

6. High-glucose (4.5 g/L) Dulbecco's Modified Essential Medium (DMEM).
7. Leibovitz L-15 medium.
8. EX-CELL® 420 Insect Serum Free medium.
9. Glutamine.
10. Gentamicin.
11. Penicillin/Streptomycin 100×.
12. Fetal calf serum (FCS). Aliquots of 50 mL are stored at –20 °C.
13. Standard medium for HeLa cell culture: DMEM (**item 6**) was supplemented with 5 mM L-glutamine (**item 9**), 5 µg/mL gentamycin (**item 10**), and 10% FCS (**item 12**). Prepare a 500 mL bottle under sterile conditions and store at 4 °C.
14. Standard medium for adult rat ventricular cardiomyocytes was 10 mM HEPES buffer with 0.3 mM Ca^{2+} and 0.5% bovine serum albumin stored at 4 °C.
15. Standard medium for C2C12 cell culture: DMEM (**item 6**) was supplemented with 5 mM L-glutamine (**item 9**), 5 µg/mL gentamycin (**item 10**), and 20% FCS (**item 12**). Prepare a 500 mL bottle under sterile conditions and store at 4 °C.
16. Standard medium for Pac2 cells: Leibovitz L-15 medium (**item 7**) was supplemented with 15% FCS (**item 12**) and 1% penicillin–streptomycin (**item 11**) from the 100× stock solution and stored at 4 °C.
17. Standard medium for Sf9 cells: EX-CELL® 420 Insect Serum Free medium (**item 8**) was supplemented with 5 mM L-glutamine (**item 9**) and 10% FCS (**item 12**) and stored at 4 °C.
18. Phosphate buffered saline (PBS) supplemented or not with 0.5 mM EDTA (PBS/EDTA).
19. HEPES buffer 1 M, pH 7.2–7.5.
20. 0.25% trypsin in PBS/EDTA prepared from trypsin powder.

2.2 Peptides

Deca-arginine peptides containing amino-terminal fluorescein label (FITC-R_{10}) were synthesized using D-amino acids by Peptide Specialty Laboratories (Heidelberg, Germany). After synthesis the peptides were HPLC purified and their molecular weight was confirmed by mass spectrometry.

Ultrapure water is used to resuspend the lyophilized peptides.

2.3 Chamber Systems and Coverslips for Live Cell Labeling of Nucleoli

1. µ-dish 35 mm low with Ibidi standard bottom (Ibidi®).
2. Chambered coverslip µ-slide 8 well (Ibidi®).
3. Chambered coverglass 4 and 8 well (Thermo Fisher Scientific, Nunc™ Lab-Tek™).
4. Coverslips, 12 mm diameter.
5. Parafilm®.

2.4 Microscopy

2.4.1 Confocal Laser Scanning Microscope

For live cell microscopy any suitable confocal microscope system can be used. We use a Zeiss LSM510Meta confocal setup mounted on an Axiovert 200 M inverted microscope equipped with a 63× phase contrast plan-apochromat oil objective NA1.4. During all acquisitions, the main beam splitter was a HFT UV/488/543/633 and the parameters for the detection of FITC were as follows:

Excitation wavelength: 488 nm.

Emission filter: BP500-530 nm.

2.4.2 Environmental Chamber and Stage Incubation System

For live cell microscopy any suitable microscope incubation system can be used. The microscope is housed in an environmental cage incubator, which is connected to a heating system providing a constant temperature of 37 °C. In addition, a chambered stage incubation system is used that provides a temperature of 37 °C and humidified 5 % CO_2 atm (all incubation components from Okolab, Ottaviano, Italy). Cells grown in chambered coverslip systems are kept inside the stage incubation system during imaging to maintain stable environmental conditions.

2.5 Image Analysis

For the analysis of microscopic images any suitable software package can be applied. We used ImageJ (http://imagej.nih.gov/ij/) to measure, e.g., relative fluorescence intensities in nucleoli compared to the nucleoplasm.

3 Methods

3.1 Cell Culture

3.1.1 Maintaining Mammalian Cells in Culture

1. HeLa and C2C12 cells (**items 1** and **3** in Subheading 2.1) are grown in p100 dishes in the cell culture incubator at 37 °C and 5 % CO_2 atm, until they reach 70–80 % confluence. Adult rat ventricular cardiomyocytes primary cells (item 2 in Subheading 2.1) cannot be maintained in culture and are used the day they are isolated.
2. Cells are grown in standard medium as indicated in Subheading 2.1.
3. To split cells, aspirate medium and rinse with 1× PBS/EDTA prewarmed to 37 °C.
4. Directly apply 1.0 mL of Trypsin–EDTA (**item 20** in Subheading 2.1) solution prewarmed to 37 °C to the cell layer and tilt the dish gently. Leave flask in the incubator for approximately 1–3 min.
5. Hit the side of the dish with the palm of one hand several times to detach the cells, avoid spilling of fluid to the side and lid of the dish.

6. When all cells have detached, add 9.0 mL of growth medium to the flask and resuspend the cells by gentle pipetting up and down several times. Avoid creating bubbles and foam. Transfer between 1.0 (1:10) and 2.0 (1:5) mL to a new cell culture dish.
7. Fill up to a final volume of 10 mL with standard medium.
8. Place the dish in a cell culture incubator and examine the cells daily using a cell culture microscope.

3.1.2 Maintaining Fish Cells in Culture

1. Pac2 cells (**item 4** in Subheading 2.1) are grown in p25 flasks in a cell culture incubator at 28 °C without CO_2 enriched atmosphere.
2. Pac2 cells are grown in standard medium (**item 16** in Subheading 2.1).
3. To split cells, aspirate medium and rinse twice with 1× PBS/EDTA (**item 18** in Subheading 2.1).
4. Directly apply sufficient trypsin–EDTA solution (**item 20** in Subheading 2.1) to cover the cell layer, tilt the dish gently, and incubate for 5 min at room temperature.
5. Dilute the detached cells with standard medium and pipette up and down several times to completely detach all cells and disperse cell clots.
6. Transfer 20% of the volume to a new flask and fill up with standard medium and place in the incubator at 28 °C.

3.1.3 Maintaining Insect Cells in Culture

1. Sf9 cells (**item 5** in Subheading 2.1) are maintained in p25 flasks as suspension culture in an orbital shaker with ambient atmosphere at 28 °C.
2. For labeling experiments with microscopic observation, cells are grown in an incubator with ambient atmosphere at 28 °C.
3. Sf9 cells are grown in standard medium (**item 17** in Subheading 2.1).
4. To subculture cells, resuspend cells gently by using a plastic pipette and transfer 1.0 mL to a new flask, fill up with standard medium and place in the orbital shaker at 28 °C.

3.1.4 Preparation of Peptides for the Labeling of Live Cells

1. Peptides are delivered lyophilized and therefore have to be resuspended.
2. Add sterile deionized ultrapure water to the lyophilized peptides to prepare peptide stock solutions at a final concentration of 1 mM, vortex to completely dissolve and homogenize the peptide solution.
3. Spin down peptide solution and prepare aliquots of 50 µL in sterile Eppendorf tubes and store at −20 °C until further use.

3.2 Preparation of Cells and Peptides for Nucleolar Labeling and Imaging

3.2.1 Culturing Cells in Live Cell Chamber Systems

1. Take live cell chambers out of sterile packaging and place into an empty sterile p100 dish (*see* **Note 1**).
2. Wash, trypsinize, and resuspend HeLa and C2C12 cells as described in Subheading 3.1.1.
3. Wash, trypsinize, and resuspend Pac2 cells as described in Subheading 3.1.2.
4. Use directly the suspension culture of Sf9 cells as described in Subheading 3.1.3, pipette up and down to disperse cells.
5. Seed equal volumes of cell suspension into each well of a sterile live cell chamber system at 60–70% confluence.
6. Typically 100 μL of cell suspension were seeded into one well of an 8-well LabTek or Ibidi chamber and correspondingly 200 μL were seeded into 4 well chambers. These amounts correspond to a split ratio of around 1: 2.5 taking into account the dish and chamber surface areas with cells grown to 70–80% confluence. Amounts of cells seeded have to be adapted to the respective cell type that will be used.
7. Fill up chambers up to half of the volume with growth medium and close the lid. To avoid contamination on the way to the incubator, close additionally the p100 dish containing the live cell chamber.
8. Human HeLa, mouse C2C12, and Pac2 fish cells are grown under the conditions described in Subheadings 3.1.1 and 3.1.2, respectively. Sf9 insect cells, however, are grown in an incubator with ambient atmosphere at 28 °C without shaking to allow attachment of the cells to the chamber surface.
9. Cells are grown over night and used the next day for nucleolar labeling with the transducible peptide marker (*see* **Note 2**).

3.2.2 Preparation of Coverslips for Fixation of Cells After Labeling the Nucleoli

1. Place 12 mm coverslips in a dedicated holder inside a closed container and immerse in pure 100% ethanol for several hours.
2. Use fine tip forceps to place the desired number of coverslips into an empty sterile cell culture dish.
3. Leave the dish with the lid half open inside the laminar flow cabinet until all traces of ethanol evaporated.
4. Immediately before starting to split cells to seed onto coverslips, use 1× PBS and fill carefully into the dish with the coverslips until all coverslips are covered. Avoid coverslips to adhere to each other by applying PBS slowly and carefully at the side of the dish without moving the coverslips.
5. Aspirate PBS and continue with washing, trypsinizing, and resuspending cells as described in Subheadings 3.1.1, 3.1.2, and 3.1.3, respectively.

6. Seed the required volume of resuspended cells to reach 70% confluence and fill up with growth medium. Avoid disturbance to prevent coverslips floating around.
7. Place the dish inside the cell culture incubator at 37 °C and 5% CO_2 until use, typically on the next day.

3.2.3 Labeling of the Nucleoli in Living Cells Using a Cell-Penetrating Peptide Marker (See Fig. 1)

1. Dilute deca-arginine peptides in DMEM without FCS to a final concentration of 10 μM just before the start of the labeling experiments and keep at 4 °C until use (*see* **Notes 3** and **4**).
2. For imaging experiments place cells grown in chambered coverslips or microscopy dishes inside the stage incubation system, fix using metal clips or similar devices to prevent movements and leave for at least 30 min to adapt to the environment and observe cells by phase contrast microscopy.
3. Remove all growth medium from the first well to be labeled and immediately apply with great care the peptide in DMEM solution (*see* **Note 5**).
4. Close the chamber lid and the stage incubation system. Adjust the microscope objective to focus the cells. It is possible to observe cells at this stage by microscopy; however, excess labeled peptide and the high concentration of fluorophores in the medium will cause very high intensities when observing the peptide fluorescence.
5. Incubate the cells for 30 min to 1 h with the peptides in DMEM. Aspirate all peptide solution, wash cells one time in DMEM without FCS or alternatively in 1× PBS to remove excess fluorescent peptides, aspirate the washing solution, and apply the standard growth medium to the cells.
6. Close the chambered coverslip and the stage incubation system, focus the cells, and start the experimental observations and image acquisition.
7. For cells grown on coverslips, aspirate medium carefully from the dish and apply the peptides in DMEM at the side of the dish, not directly on the coverslips (*see* **Note 6**). After removal of the peptide in DMEM solution use forceps to place the coverslips with cells turned upward on Parafilm in a humidified chamber for further treatment.
8. Perform the washing steps with 1× PBS on individual coverslips in the humidified chamber and proceed with a protocol for cell fixation, permeabilization, and immunostaining (*see* **Note 7**).
9. Alternatively, the application of peptides in DMEM, as well as the washing step can be performed in a laminar flow biosafety cabinet. In this case, cells are kept in the cell culture incubator for the peptide uptake incubation. Microscopic observation can start after labeling or any time later (*see* **Note 8**).

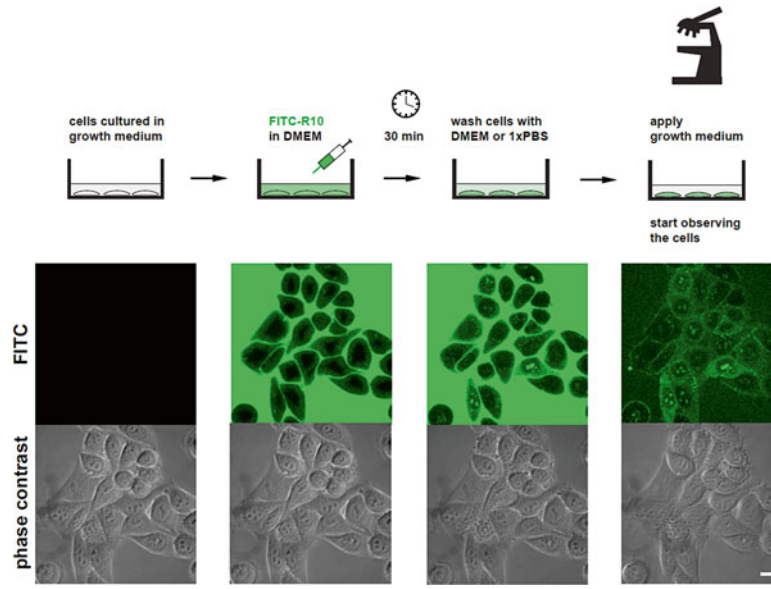

Fig. 1 Application of a fluorescent cell-penetrating-peptide-based nucleolar marker. The *upper row* shows schematically the steps for the labeling of nucleoli in live cells with the cell-penetrating peptide marker. Cells are cultivated in microscopy dishes and after removal of the growth medium incubated with the peptide marker diluted in DMEM. After an incubation time of about 30 min, the peptide solution is removed and cells are washed with fresh DMEM or PBS to remove excess fluorescent peptides. Finally the cells are supplied with standard growth medium followed by immediate microscopic observation of the fluorescently labeled nucleoli. The *lower panel* shows exemplary images of a culture of human HeLa cells at the different stages of the labeling process depicted in the scheme earlier. The *upper image row* shows FITC fluorescence of the cell-penetrating peptide marker with the corresponding phase contrast image below. Note the lack of signals inside the cell after application of the fluorescent peptide in the second step, while after 30 min of incubation in step 3, nucleolar labeling is already visible. After washing away excess peptide, the fluorescently labeled nucleoli are distinctively visible in the vast majority of cells against the darker nucleoplasmic surrounding. Scale bar 10 μm

10. For cells in suspension culture, we suggest mild centrifugation steps to remove growth medium, resuspension of cells in DMEM with diluted peptides followed by mild centrifugation for peptide removal and washing steps.
11. Additional labeling of cells with cell permeable dyes like DRAQ5 [21] can be performed before or after peptide labeling of nucleoli (*see* **Note 9**).

3.3 Imaging (See Fig. 2)

1. Place the dish containing the cells treated with the nucleolar marker on the microscope stage. Close the incubation chamber and wait 30–60 min to allow the sample to acclimatize to the chamber temperature before starting with image acquisition. This will avoid focus drift while imaging.

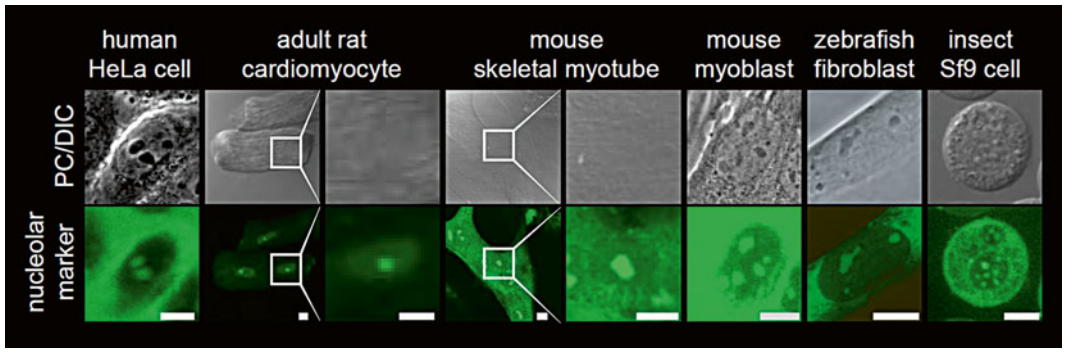

Fig. 2 Intracellular distribution of nucleolar markers in living cells of various species and origin. Shown are mid-optical sections of cultured cell lines and terminally differentiated primary cells as indicated earlier. For visualization of nucleoli in situ, cells were treated for 30 min with medium containing the nucleolar marker, washed in PBS, and incubated in medium for live cell imaging on a confocal microscope equipped with a climatization chamber to maintain a constant temperature of 37 °C, 5 % CO_2, and 60 % humidity. The *lower image row* shows FITC fluorescence of the cell-penetrating peptide marker with the corresponding phase/differential interference contrast (PC/DIC) image below. Scale bar 10 μm

2. Focus the cells using the transmitted light channel (i.e., differential interference contrast (DIC), phase contrast, etc.).
3. Adjust the microscope settings, in particular, the 488 nm laser wavelength intensity and exposure time to optimize the visualization of the nucleoli (minimizing overexposure of the sample). To capture the entire volume of the nucleolus it is required to image optical sections that span approximately −4 μm to 7 μm from the focal plane (for HeLa cells, *see* **Note 10**).
4. Image the cells.
5. Export the images as tiff files for further processing using ImageJ.

3.4 Image Analysis (See Fig. 3)

Segmentation of Nucleoli

1. Open the image stack (or single image) in ImageJ.
2. Select the cell of interest using the "rectangular" selection option in the tools menu bar.
3. Crop ("Image" menu > "Crop"), Fig. 3a.
4. Separate the stack into single images ("Image" menu > "Stacks" > "Stacks to Images"), Fig. 3b.
5. Close without saving the images that do not contain any fraction of the nucleus. The marker distributes strongly in the cytosol and nucleoli and is weakly distributed over the nucleoplasm. This can be used to easily recognize the nucleus without the need of any extra nuclear marker.

For Each Stack Image

6. Select the nucleus using the "Freehand selections" in the tools menu bar, Fig. 3c.

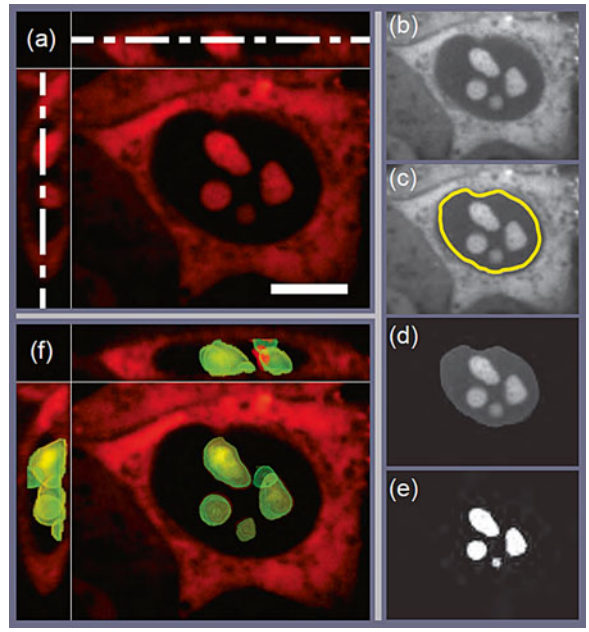

Fig. 3 Image analysis steps for segmentation of nucleoli. (**a**) Image stack of a single HeLa cell visualized using Volocity. *xy* as well as *xz* and *yz* views of the cell are shown. (**b**) Example image from z-stack for segmentation. (**c**) Nucleus selection (*yellow line*). (**d**) Clearance of signal outside the nucleus. (**e**) Binary image of the nucleoli. (**f**) Overlay of image stack in (**a**) and a mesh representation of the surface of the 3D rendered nucleoli. Scale bar 10 μm

7. Clear the marker signal outside the nucleus ("Edit" menu > "Clear Outside"), Fig. 3d.
8. Convert the image to a binary form to isolate the nucleoli region ("Process" menu > "Binary" > "Make Binary" and use the "Otsu" filter (*see* **Note 11**), Fig. 3e).
9. For further processing each image can be used individually as a mask for the nucleolus or the images can be collected into a single stack ("Image" menu > "Stacks" > "Images to Stacks").
10. Save resulting individual images or image stack as tiff (click selected image and "File" menu > "Save as" > "tiff").
11. The 3D stack can be visualized using the ImageJ plug-in "3D Viewer" ("Plugins" menu > 3D > "Volume Viewer"), select view angle and options and save as an image "Save View," Fig. 3f.

4 Notes

1. We place the chambered coverslip systems and microscopy dishes inside p100 cell culture dishes for better handling, easy transport, and sterility between laminar flow biosafety cabinet, incubator, and microscope incubator.

2. For primary cells, e.g., freshly isolated cardiomyocytes it is recommended to perform the peptide labeling and washing steps as soon as possible after preparation of the sample due to their very limited lifespan [20]. In the case of other primary cell cultures with longer survival time, it might be possible to delay the labeling after sample preparation.
3. The efficiency of uptake of cell-penetrating peptide markers is a function of peptide length and concentration but is also cell type specific. A concentration of 10 μM FITC labeled deca-L-arginine was sufficient to label a range of different cell types from different species (*see* Fig. 2 and [18]). However, for certain cell types the concentration of FITC-R_{10} might be elevated up to 50 μM to achieve efficient nucleolar labeling with a low to moderate rate of toxicity [22]. Higher concentrations of R_{10} peptide might result in high frequency of damaged or dead cells.
4. For the peptide transduction we prepare a volume of peptides diluted in DMEM sufficient to completely cover all cells in the respective number of chambers to be labeled. Typically in a single well of a 4-well chamber it is sufficient to apply 200 μL of diluted peptide in DMEM to cover all cells completely and provide enough volume to prevent dry spots in the center of the well due to the fluid meniscus. Accordingly, a volume of 100 μL is sufficient in one well of an 8-well chamber.
5. When applying the peptide diluted in DMEM to the cells, place the tip of the pipette into one corner of the well, close to the bottom but not touching it and slowly release the fluid to immerse the cells. Avoid drops of liquid falling on the cells to prevent detaching of cells. When the chamber bottom surface is covered with liquid, add the rest of the volume slightly faster but still with great care. Perform washing steps and the final application of growth medium in the same way.
6. For increased efficiency of peptide uptake into cells to be fixed, we incubated cells with the peptide up to 1 h.
7. Membrane permeable peptides are not recommended for labeling of nucleoli postfixation [20].
8. The persistence of the nucleolar labeling in living cells was successfully observed up to 24 h after the labeling event.
9. For additional labeling of cellular components by transient transfection of cells with, e.g., plasmids encoding proteins tagged with red fluorescent proteins, we suggest to perform the transfection the day before the nucleolar labeling with the peptides.
10. This range depends significantly on the specific cell type and stage of the cell cycle.
11. Most filters (e.g., “MaxEntropy,” “Mean,” etc.) can be used here with very similar results.

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