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Easy detection of primary cilia by immunofluorescence

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Dear Editor,

We thank you for the comments and suggestions made by the reviewers. They were valuable and helpful in the improvement of our manuscript. We have made the required revisions according to the referees' comments and suggestions.

With best regards,

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TITLE:

Simple Detection of Primary Cilia by Immunofluorescence

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KEYWORDS:
primary cilia, immunofluorescence, fibroblasts, acetylated alpha tubulin, gamma tubulin, microscopy, cultivation in vitro, serum starvation, irradiation, doxorubicin, taxol

SUMMARY:
Primary cilia are extracellular structures associated with the centriole. Primary cilia detection by immunofluorescent staining is a relatively simple procedure that results in extremely high-quality images. In this protocol, fibroblasts expressing primary cilia were fixed, immunostained, and imaged in a fluorescent or confocal microscope.

ABSTRACT:
Primary cilia are dynamically regulated during cell cycle progression, specifically during the G0/G1 phases of the cell cycle, being resorbed prior to mitosis. Primary cilia can be visualized with highly sophisticated methods, including transmission electron microscopy, 3D imaging, or using software for the automatic detection of primary cilia. However, immunofluorescent staining of primary cilia is needed to perform these methods. This publication describes a protocol for the easy detection of primary cilia in vitro by staining acetylated alpha tubulin (axoneme) and gamma tubulin (basal body). This immunofluorescent staining protocol is relatively simple and results in high-quality images. The present protocol describes how four cell lines (C2C12, MEF, NHLF, and

skin fibroblasts) expressing primary cilia were fixed, immunostained, and imaged with a fluorescent or confocal microscope.

INTRODUCTION:

Primary cilia are sensory, solitary, membrane-bound, nonmotile structures associated with the cell's mother centriole. Primary cilia are found on most vertebrate cells with the exception of red blood cells, adipocytes[1], and hepatocytes[2]. Primary cilia are formed as an elongated axoneme composed by microtubules, whose main component is α-tubulin. The axoneme grows from the basal body, which is structured from γ-tubulin. The length of the primary cilia varies between 2–10 µm; however, its dimensions can change during glycylation, starvation, hypoxia, cytotoxic stress, or after exposure to ionizing radiation[3–7]. Usually, cells have only one primary cilium, which is involved in morphogenesis and cell signalling pathways important for cell proliferation and differentiation[8,9].

Primary cilia are dynamically regulated during cell cycle progression, specifically during the G0/G1 phases, and resorbed before entering mitosis in a process associated with tubulin deacetylation mediated by HDAC6 (histone deacetylase 6)[10]. The exact moment of primary cilia resorption depends upon cell type and the expression of genes directly involved in this process, such as *Aurora A, Plk1, TcTex-1*[11–13]. Depending on the cell type, the primary cilia express different types of receptors, ion channels, and active signalling pathways. These include the most important signalling receptors affecting proliferation and survival, EGFR, PDGFR, and FGFR. Also included are some of the signalling pathways that may affect the function of one or more organs, including Hedgehog, Notch, and Wnt. Thanks to these receptors and signalling pathways, the primary cilia also perform a chemosensory function. This function allows primary cilia to detect specific ligands for Notch, hormones, and biologically active substances such as serotonin or somatostatin. Other specific functions exhibited by primary cilia of different lengths include reaction to changes in temperature, gravity, and osmolality[14].

Primary cilia can be visualized through various methods, such as live visualization, transmission electron microscopy, 3D imaging, or by software for the automatic detection of primary cilia[5,15–17]. However, these methods are highly specialized and ongoing research needs basic, fast, and easy methods for staining primary cilia in every stage of research. Described is an easy and useful method for the detection of primary cilia in cultured cells.

PROTOCOL:

1. Preparation of culture media, solutions, and dishes

1.1. Autoclave the coverslips (22 x 22 mm). Prepare 6 well plates. Thaw fetal bovine serum (FBS) and antibiotic penicillin/streptomycin and warm the culture medium to room temperature (RT). Use trypsin-EDTA (0.25%) and 1x PBS (phosphate buffered saline with calcium and magnesium to passage the cells.

1.2. Prepare fresh 4% paraformaldehyde (PFA) in dH_2O (800 mg of PFA in 20 mL of dH_2O). The

PFA must be freshly prepared for each experiment. Stir and heat the solution at 55 °C for 30 min in the hood. Cool down at RT. Add 1 M sodium hydroxide until the solution becomes clear (pH = 7.2–7.4). Store at 4 °C for up to 1 week.

Note: PFA is toxic; always wear adequate personal protective equipment and prepare in the chemical hood.

1.3. Prepare 500 mL of culture media, DMEM (Dulbecco´s Modified Eagle´s medium) containing 10% FBS, 1% penicillin/streptomycin, and 2% glutamine.

1.4. Prepare 13 mL of 1% gelatin solution in sterile dH_2O (130 mg of gelatin in 13 mL of dH_2O). Use 2 mL of 1% gelatin for each well in a 6 well plate. Keep sterile.

1.5. Clean the laminar flow hood using 70% ethanol. Place the required material inside the laminar flow hood before starting the experiment.

2. Cell culture for immunocytochemistry staining

2.1. Thaw the cells (in this study C2C12, MEF, NHLF, and skin fibroblasts) using standard techniques and plate them in a T75 flask supplemented with ~10–12 mL of the prepared media. Incubate at 37 °C/5% CO_2/90% relative humidity (RH) until the cells reach 70% confluence.

2.2. Remove the cells from the incubator and place them in the laminar flow hood. Remove the culture media and rinse the cells briefly 2x with 1x PBS. Add ~2 mL of 0.25% trypsin-EDTA into the T75 flask and incubate at 37 °C for ~5 min. Check periodically on the inverted microscope to monitor cell detachment.

NOTE: The incubation time depends on the cell line and therefore must be determined empirically.

2.3. Gently resuspend the cells in 10 mL of culture media, pipetting carefully to create a single cell suspension. Rinse the flask again if necessary.

2.4. Place the cell suspension in a 50 mL conical tube and centrifuge for 5 min at ~200 x *g*. Decant the supernatant, add 10 mL of culture media, and gently resuspend the pellet. Take 20 µL of the cell suspension and mix in a 1:1 ratio with trypan blue and count in a cytometer following the standard method.

2.5. Place one coverslip inside each well of a 6 well plate using tweezers. Coat the coverslips with gelatin by pouring ~2 mL into the wells. This will help the cells attach to the coverslips. Remove the gelatin solution and let air-dry for a few minutes. The coverslips are now ready for cultivation of the cells. Start the cultivation immediately.

2.6. Seed 100,000 fibroblasts into each well and add 2 mL of culture media. Incubate the cells for

24 h at 37 °C/5% CO_2/90% RH. At this point, the cells can be treated according to the needs of the user. Treatments to induce ciliation have been previously described[4,5].

NOTE: The initial seeding number depends on the cells' doubling time and should be determined accordingly.

3. Immunofluorescent staining of primary cilia in vitro

3.1. Warm the 4% paraformaldehyde to RT. Prepare Pasteur pipettes, 1x PBS (RT), waste container, 15 mL conical tubes, micropipettes (0.5–10 µL, 20–200 µL, and 100–1,000 µL) and tips. Take the cells from the incubator and place them in the bench.

NOTE: The staining procedure does not need to be performed in sterile conditions. All solutions must be at RT.

3.2. Remove media from each well. Leave the coverslip inside the well. Very gently wash the cells 3x with 2 mL of 1x PBS. Using a Pasteur pipette, add 2 mL of 4% PFA into each well to fix the cells. Incubate for 10 min at RT. Remove the PFA and wash 3x with 1x PBS.

NOTE: Always use a sufficient volume to cover the entire coverslip during the incubation periods. Never let the cells dry. Never pour any of the solutions directly onto the coverslip.

3.3. Prepare 0.5% Triton X-100 in 13 mL of 1x PBS 10 min before use. Add 2 mL into each well. Incubate for 15 min. Wash gently 4x with 1x PBS.

NOTE: Triton X-100 is insoluble in PBS at RT. Heat the 0.5% Triton X-100 solution to 37 °C in a water bath to dissolve it.

3.4. Thaw goat serum 5 min before use. Dilute the goat serum in 1x PBS in a 1:20 ratio as a blocking solution. Add 150 µL to each coverslip and incubate for 20 min at RT.

NOTE: Prolong the blocking period up to 60 min if necessary. Do not wash the cells after blocking with goat serum.

3.5. Thaw the primary antibodies (i.e., anti-acetylated alpha tubulin and anti-gamma tubulin) 5 min before use. Dilute the antibodies separately in 1x PBS as follows: mouse anti-acetylated alpha tubulin in a 1:800 ratio and rabbit anti-gamma tubulin in a 1:300 ratio. Remove the blocking solution. Do not wash. Add 150 µL of both antibody dilutions to the coverslips and incubate for 60 min at RT.

NOTE: If incubating overnight use 500–1,000 µL of the primary antibody solutions, seal the 6 well plate with paraffin film, and store at 4 °C. Alternatively, use 150 µL of antibody and incubate in a humidity chamber.

3.6. Remove the primary antibodies. Wash the coverslips very gently 3x with 2 mL of 1x PBS. Prepare the secondary antibodies in 1x PBS by separately diluting Cy3 sheep anti-mouse and Alexa Fluor488 goat anti-rabbit in a 1:300 ratio. Add 150 µL of both secondary antibody dilutions to the coverslips. Incubate for 45 min at RT in the dark.

NOTE: Incubate in the dark to avoid photobleaching. Other combinations of secondary antibodies can be used as needed.

3.7. Prepare a DAPI (4', 6-diamidino-2-phenylindole) solution according to the manufacturer's instructions. Store the excess aliquots at -20 °C. Dilute 10 µL from a stock aliquot (1:5,000) in 50 mL of 1x PBS. Add 2 mL of this dilution to the coverslips. Incubate for 5 min at RT in the dark.

NOTE: It is important to incubate the cells in the dark to avoid photobleaching. The DAPI dilution can be stored at 4 °C for up to 1 month.

3.8. Prepare 2 needles, slides, tweezers, and mounting media. Label the slides.

3.9. Remove the DAPI solution from the wells. Wash 3x with 1x PBS. Put one drop of mounting media on each slide. Use the needle to gently lift the coverslip from the well's bottom. Flip the coverslip using the tweezers and gently place it over the drop of mounting media. Carefully remove any bubbles.

3.10. Protect the slides from light and store them overnight at 4 °C.

3.11. Use a fluorescent or confocal microscope with high magnification to visualize the primary cilia.

NOTE: The slides can be stored in the dark at 4 °C for up to 2 months.

REPRESENTATIVE RESULTS:

The immunofluorescent staining of primary cilia is a relatively simple procedure that results in high-quality images. In these experiments, fibroblasts expressing primary cilia were fixed, immunostained, and imaged in a fluorescent or confocal microscope following the protocol described above. The primary cilium was detected using acetylated α-tubulin and γ-tubulin. The evaluation of primary cilia can be performed on various levels and any change in this regard can be linked to exposure to ionizing radiation, cell metabolism (e.g., starvation), or chemical treatment (e.g., cytostatics)[5,18].

The effect of ionizing radiation on primary cilia has been studied in various cell lines (e.g., the myoblast cell line C2C12), which were irradiated (2, 6, 10, and 20 Gy) and the changes in primary cilia incidence analyzed. According to Filipova at al.[4], low irradiation doses do not modify the occurrence of a single primary cilia in C2C12 cells. However, higher doses of ionizing radiation (i.e., 20 Gy) induced the appearance of multiple primary cilia (**Figure 1A,B,C**). Similarly, when NHLF cells were irradiated at 2 Gy the primary cilia were detected by immunofluorescence

(**Figure 2**).

Metabolic stress is also known to increase the frequency of primary cilia[19]. In this case, MEF fibroblasts were starved and analyzed for changes in primary cilia incidence (**Figure 3**).

Immunofluorescence staining revealed that fibroblast cells carried primary cilia after treatment with doxorubin and taxol. Those fibroblasts treated with 120 nM doxorubicin expressed a single primary cilium (**Figure 4**); higher doses induced the appearance of multiple primary cilia (**Figure 5**). Treatment with 1.25 nM taxol also resulted in the presence of a single primary cilium (**Figure 6**). In contrast to the treatment with doxorubicin, multiple cilia were not detected after treatment with higher doses of taxol[5].

FIGURE AND TABLE LEGENDS:

Figure 1: Occurrence of primary cilia in irradiated C2C12 cells. Representative photographs of primary cilia in C2C12 cells. Primary cilia detection was performed by immunofluorescence. The axoneme (arrow) of the primary cilia were assessed with acetylated α-tubulin antibody (red) and the basal body by γ-tubulin antibody (arrow, green). Nuclei were stained with DAPI (blue). (**A**) and (**B**) multiple cilia were observed 72 h after irradiation with 20 Gy. (**C**) Single primary cilia after 72 h irradiation with 20 Gy[4].

Figure 2: Detection of primary cilia in irradiated NHLF cells. Representative photographs of primary cilia in NHLF cells. Primary cilia (arrow) detection was performed by immunofluorescence. The axonemes of the primary cilia were stained with acetylated α-tubulin antibody (red) and the basal bodies with γ-tubulin antibody (green). Nuclei were stained with DAPI (blue). Single primary cilia 24 hours after irradiation at 2 Gy.

Figure 3: Incidence of primary cilia in the MEF cells after metabolic stress induced by serum starvation. Representative photographs of primary cilia 24 h after serum starvation (0.1% FBS) in MEF cells. Primary cilia (arrow) detection was performed by immunofluorescence. Axonemes were labeled with acetylated α-tubulin antibody (red). Basal bodies were stained with γ-tubulin antibody (green). Nuclei were stained with DAPI (blue).

Figure 4: Representative photographs of primary cilia in skin fibroblasts. Primary cilia (arrow) detection was performed by immunofluorescence. Primary cilia were stained with acetylated α-tubulin antibody (red), while the basal bodies were stained with γ-tubulin antibody (green). Nuclei were stained with DAPI (blue). Primary cilia were detected 72 h after treatment with 120 nM doxorubicin[5].

Figure 5: Representative photographs of multiple cilia in skin fibroblasts. Primary cilia (arrow) detection was performed by immunofluorescence. The axonemes were labeled by acetylated α-tubulin antibody (red) and the basal bodies were stained with γ-tubulin antibody (green). Nuclei were stained with DAPI (blue). Multiple cilia were detected 72 h after treatment with 120 nM doxorubicin[5].

Figure 6: Representative photographs of skin fibroblasts treated with taxol. Primary cilia (arrow) were detected by immunofluorescence. Primary cilia were stained with acetylated α-tubulin antibody (red) and with γ-tubulin antibody (green). Axoneme nuclei were stained with DAPI (blue). Primary cilia were detected 72 h after treatment with 1.25 nM taxol[5].

DISCUSSION:

Several authors have described diverse methods for the detection of primary cilia, sometimes also describing various fixation methods that can affect their detection[6,20–22]. Regardless, it is difficult to find a complete and straightforward protocol for detection. The ready availability of such a method would undoubtedly be of great assistance to the study of primary cilia investigation, especially in early stages of research or for a quick and easy method to test the presence of primary cilia in a chosen cell line. Therefore, this protocol is described in as much detail as possible for the detection of primary cilia in vitro after different kinds of treatment.

The present protocol was modified for use on a daily basis[20,23,24]. For example, 10% formalin was replaced by 4% PFA, whose fresh preparation is recommended due to its short storage life. PFA is a good choice for preserving cell morphology and is especially suited to the visualization of membrane-bound proteins. Organic solvents, such as methanol, have a dehydrating effect on the cell and remove small, soluble molecules and lipids during the fixation process, thus making it unsuitable for use in certain scenarios[25]. Permeabilization is achieved with 0.5% Triton X-100 in 1x PBS for 15 min. Goat serum in a 1:20 dilution in 1x PBS for 20 min is used as a blocking agent. Both primary antibodies, mouse anti-acetylated tubulin and rabbit anti-γ-tubulin, can be incubated concurrently for 60 min using a 1:800 and 1:300 dilution in 1x PBS, respectively[20,21,23,24]. In addition, the secondary antibodies, anti-mouse IgG (whole molecule) F(ab’)2 fragment–Cy3 antibody produced in sheep and Alexa Fluor488 AffiniPure $F(ab')_2$ fragment goat anti-rabbit IgG, were diluted 1:300 in 1x PBS. They were incubated concurrently for 45 min.

It may be necessary to take extra standardization steps should the primary antibodies be incubated overnight. During the development of the protocol it was found that an overnight incubation needs a volume of at least 500–1,000 µL of primary antibodies solution, the 6 well plate must be sealed with parafilm, and storage must be at 4 °C to prevent evaporation.

The most critical steps for the successful staining of primary cilia are: 1) choice of cell line and optimal cell culture practice; 2) use of gelatin coated coverslips; 3) consistent use of fresh 4% paraformaldehyde; 4) incubation of the secondary antibody and DAPI in the dark; 5) performing a gentle flip and placement of the coverslip on top of the mounting media in the slide.

There are no foreseen potential limitations in the future applications of the protocol. Moreover, primary cilia research is becoming more relevant in a variety of fields, and easy, fast, and reliable cilia detection methods are essential. Further, this protocol will facilitate the future study of primary cilia in cell types in which primary cilia have been heretofore undetected.

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DISCLOSURE:

The authors have nothing to disclose**.**

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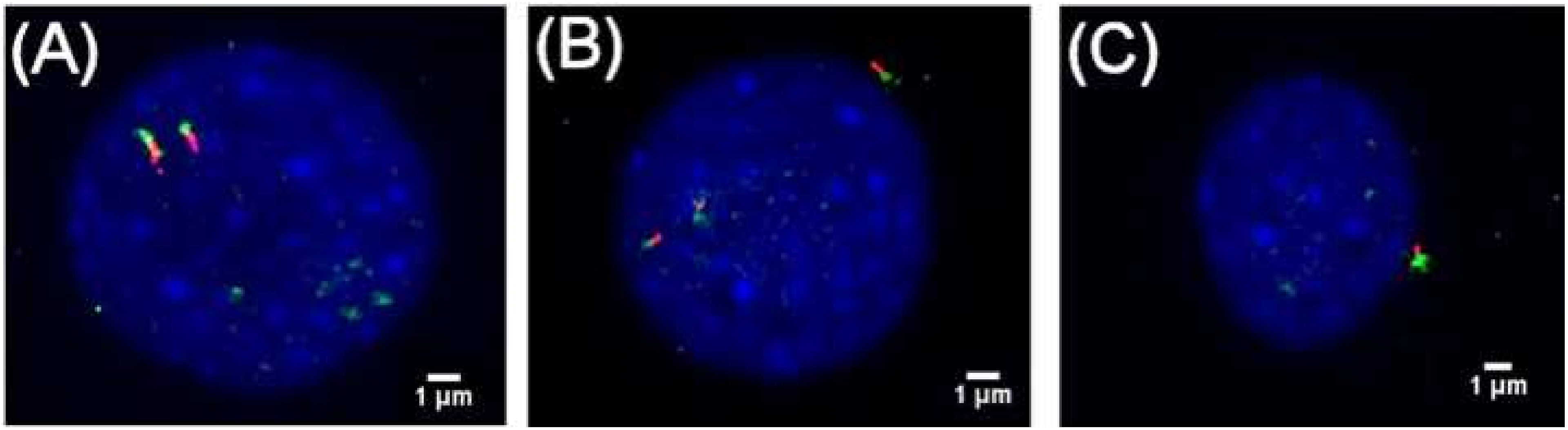
(A)
1 µm
(B)
1 µm
(C)
1 µm

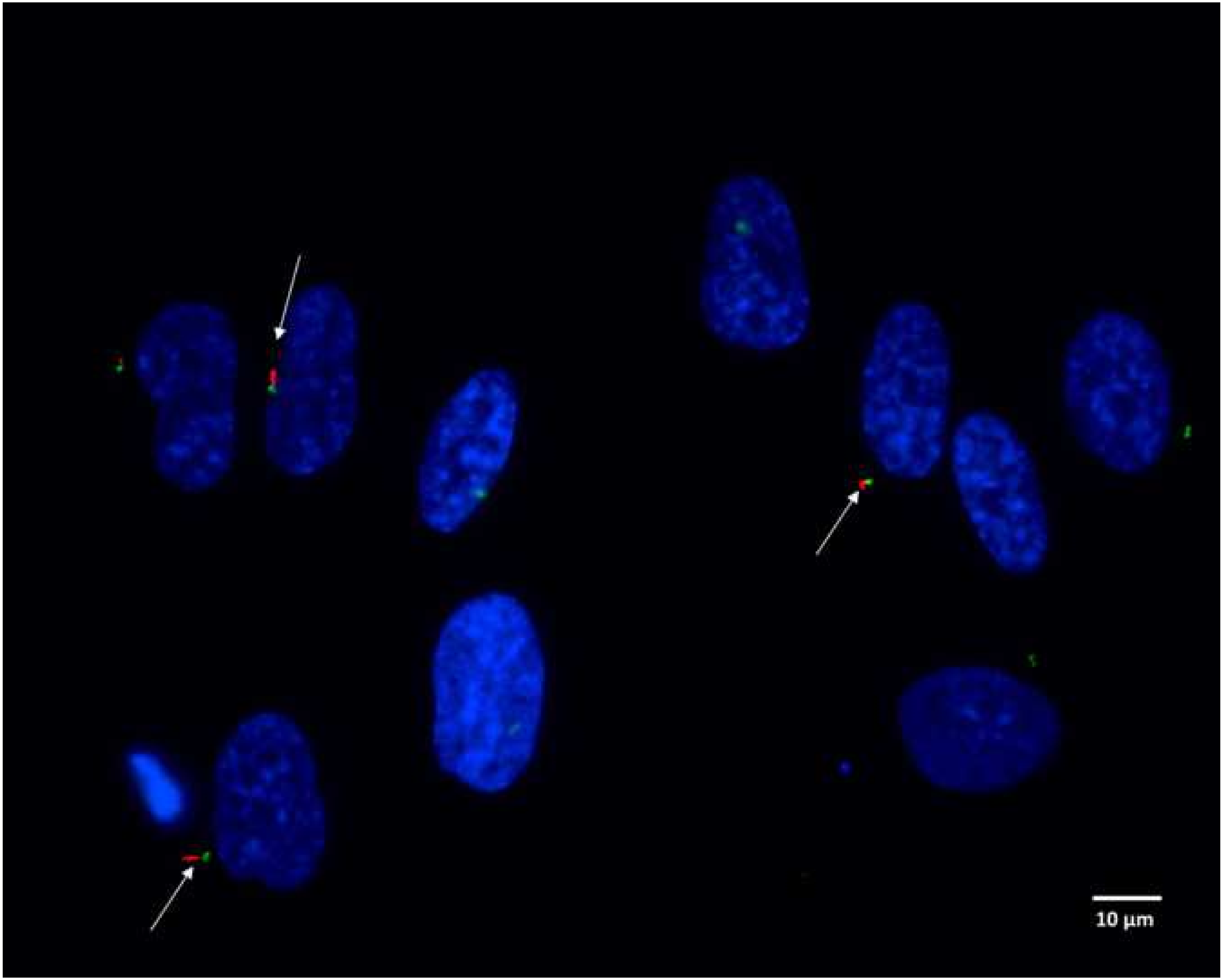
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Figure_3

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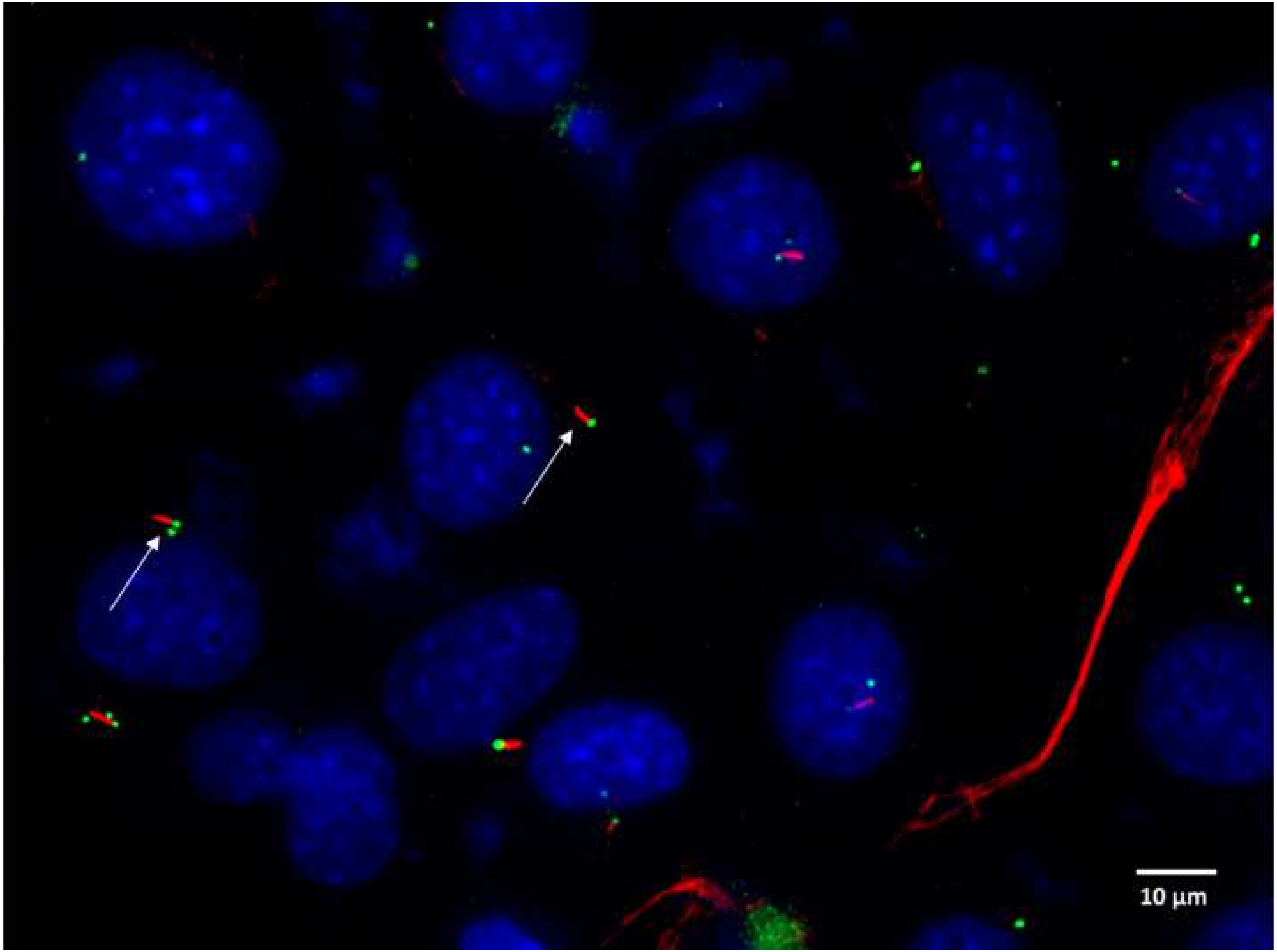

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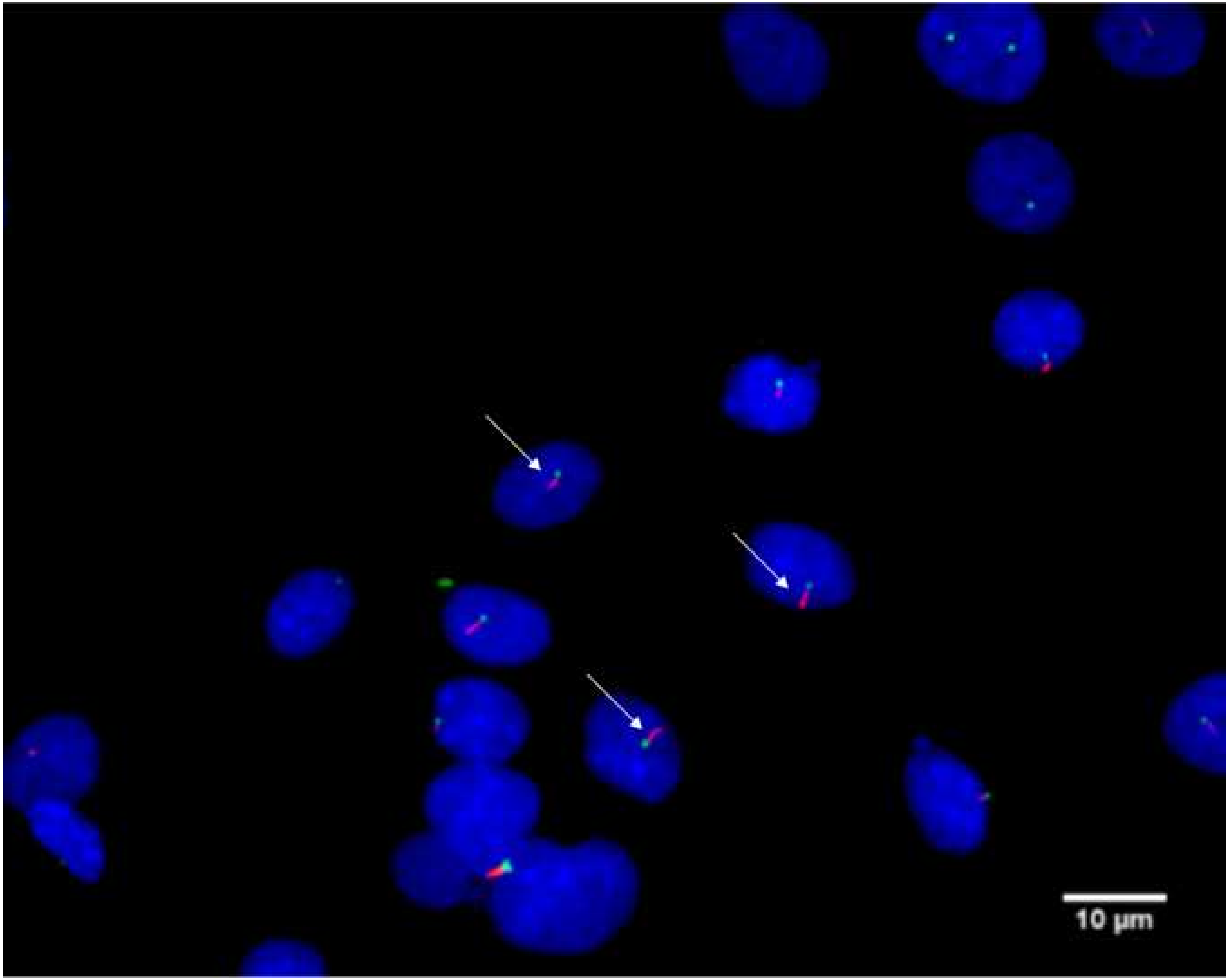
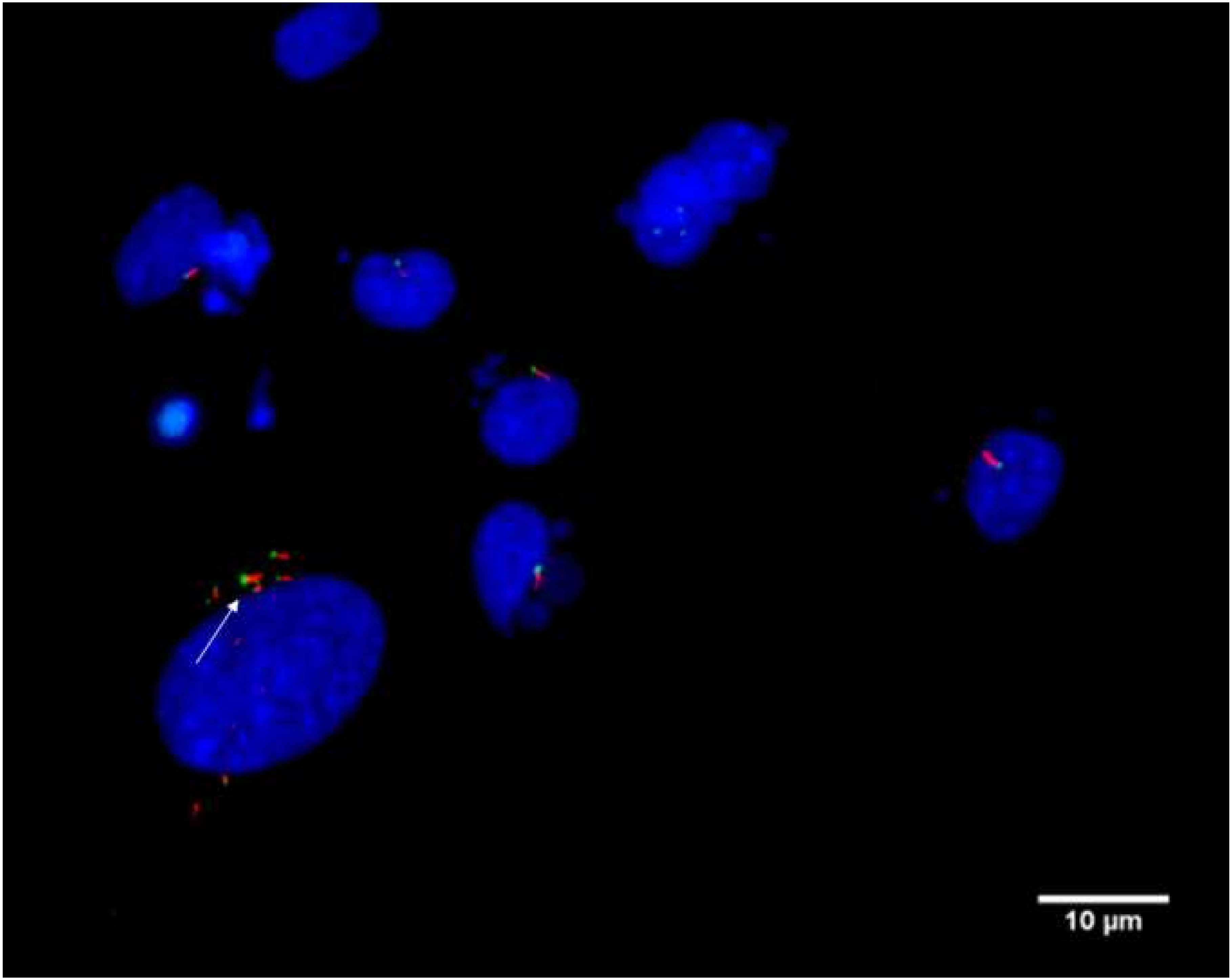
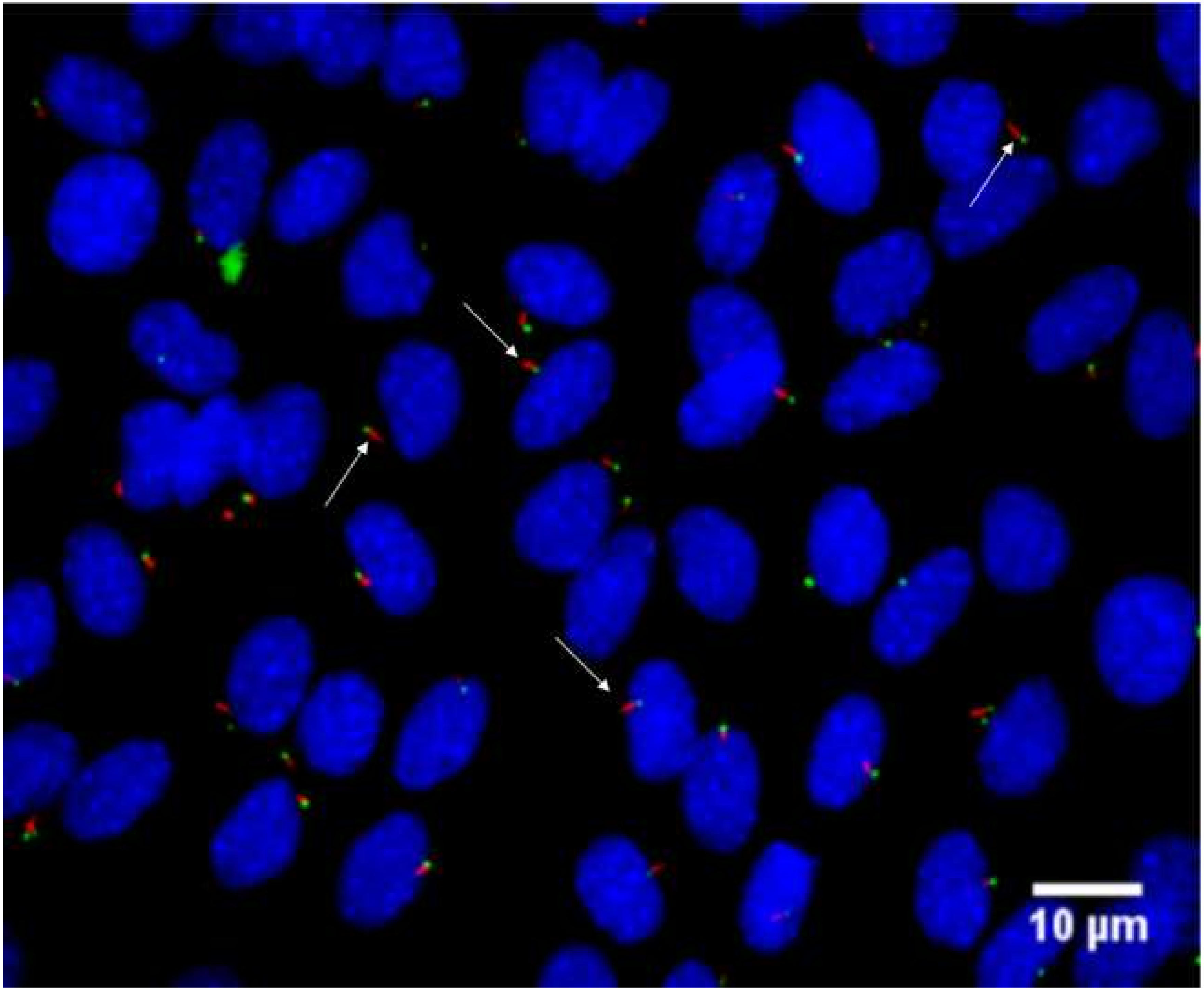


Figure_5




10 μm

Name of Material/ Equipment	Company	Catalog Number
6-well plate	TPP	92406
Alexa Fluor488	Jackson ImmunoResearch	111-546-047
Anti-Tubulin γ	Sigma-Aldrich	T5192
C2C12	ATCC	CRL-1772
Cy3	Sigma-Aldrich	C2181
Dapi (4′,6-Diamidino-2-phenylindole dihydrochloride)	Sigma-Aldrich	D9542
Dulbecco´s Modified Eagle´s medium	Thermo Scientific	11960044
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich	D8662
Fetal Bovine Serum	Thermo Scientific	16000044
L-Glutamine	Sigma-Aldrich	G7513
MEF	ATCC	SCRC-1039
Monoclonal Anti-Acetylated Tubulin	Sigma-Aldrich	T7451
NHLF	Lonza	CC-2512
Normal Goat Serum	Jackson ImmunoResearch	005-000-121
Paraformaldehyde	Sigma-Aldrich	158127-500G
Penicillin-Streptomycin	Sigma-Aldrich	P0781
ProLong Diamond Antifade Mountant	Thermo Scientific	P36961
Skin fibroblasts	Kindly gifted from Charles University, Faculty of Medicine in Hradec Králové.	
Square Cover Slips	Thermo Scientific	22X22-1.5
Triton X-100	Sigma-Aldrich	11332481001
Trypsin-EDTA (0.25%)	Thermo Scientific	25200072

Comments/Description

Dimensions 128x86x22 mm
AffiniPure $F(ab')_2$ Fragment Goat Anti-Rabbit IgG
Polyclonal Rabbit anti-Mouse IgG2a
Myoblast (mouse)
Anti-Mouse IgG (whole molecule) F(ab')2 fragment–Cy3 antibody produced in sheep

High glucose, No glutamine, Gibco
With $MgCl_2$ and $CaCl_2$, Sterile-filtered, Suitable for cell culture
Sterile-Filtered, Gibco

Mouse embryonic fibroblast
Monoclonal Anti-Acetylated Tubulin antibody produced in mouse
Primary lung fibroblasts (human)

Powder
10,000 units penicillin and 10 mg streptomycin per mL in 0.9% NaCl, Sterile-Filtered

Borosilicate glass, 22x22mm, Square

Sterile-Filtered, Gibco

RESPONSE TO REVIEWER _1

1. **No other methods of fixation, blocking or staining were compared. Is this really the best protocol if there is no comparison to other methods discussed or shown?**

   We appreciate this comment. Our protocol has been developed and optimized by studying expert articles across this topic. We don't make a comparative study.

2. **Does this protocol allow for imaging of other ciliary proteins that are currently of high-interest in the ciliary research field, such as transition zone proteins? Staining for many of these proteins requires methanol fixation.**

   Thank you for pointing this out. Our protocol is focused toward primary cilia staining (i.e. axoneme and basal body), that's why we used the PFA for fixation. We carefully reviewed several articles and some authors use methanol instead to fix transition zone proteins. However, some of them use PFA fixation e.g.:

   Schou KB et al. KIF13B establishes a CAV1-enriched microdomain at the ciliary transition zone to promote Sonic hedgehog signalling. Nat Commun. 2017 30;8:14177. doi: 10.1038/ncomms14177.

   Takao D et al. Protein Interaction Analysis Provides a Map of the Spatial and Temporal Organization of the Ciliary Gating Zone. Curr Biol. 2017 7;27(15):2296-2306.e3. doi: 10.1016/j.cub.2017.06.044.

   Kim MS et al. describing that the best fixation condition for transition zone proteins uses both PFA and methanol. It is best to test all three fixation conditions (PFA, Methanol,and both together) to determine suitability for a particular antibody. Kim MS et al. Immunofluorescent staining of septins in primary cilia. Methods Cell Biol. 2016;136:269-83. doi: 10.1016/bs.mcb.2016.03.015.

3. **Why were these cell lines chosen, and not the well characterised ciliary cell models such as mouse IMCD3, or human RPE-1 cells?**

   The cell lines were chosen based on previous experiments and well establish reaction after ionizing radiation, serum starvation and cytostatics. Also, we chose these cell lines to show readers that there are additional cell lines possessed of primary cilia and in which it is easy to promote ciliogenesis.

4. **Images are not correctly labelled with which colour represents which antibody or stain.**

   As suggested by the reviewer, we have changed the labeling of the images (see changes in the text file).

Dear referee, thank you for the time and effort set in the review of our manuscript. It surely improved its quality and impact.

RESPONSE TO REVIEWER _2

1. **It is unclear that the first sentence of the abstract fits the purpose of this protocol. The statement, "Primary cilia can be visualized through several methods, including TEM, 3D imaging or using software for the automatic detection of PC," doesn't fit this protocol. Immunofluorescence staining would still be needed to do 3D imaging or automatic detection of PC.**

   Thank you for pointing this out. We changed the sentence: " Primary cilia can be visualized through several highly sophisticated methods, including transmission electron microscopy, 3D imaging or using software for the automatic detection of primary cilia. However, immunofluorescence staining of primary cilia would still be needed to perform these methods and the detection of primary cilia still needs basic, fast and easy protocols for their staining."

2. **"Extremely high quality" is overstated based on the images presented in the figures.**

   As suggested by the reviewer, we have changed the statement to: "quality images".

3. **In the last sentence - I think "fluorescent" is supposed to mean "epifluorescence" microscope (confocal microscopy also relies on fluorescence).**

   We are grateful for this comment. However, we meant fluorescence and confocal microscopes. A fluorescence microscope allows the detection and localization of fluorescent molecules in the sample. A confocal microscope is a specific fluorescent microscope that enables the obtention of 3D images of the sample with good resolution. In both methods, the sample contains fluorescent molecules.

4. **Was "axoneme" was meant by "axonema"?**

   "Axoneme" is the correct term in the English language.

5. **The general IF portion of the protocol (fix, block/permeabilize, primary ab, wash, secondary ab, wash, mount, image) could also be applied to staining for PC in tissues - e.g. intact mouse tissues or thin sections.**

   In accordance with the reviewer's comment, the portion of the protocol can be used for staining primary cilia in paraffin embed tissue or for other experiments where fluorescence is needed.

6. **A description of the differences between PFA and methanol fixation and why PFA was chosen here would be helpful.**

   In accordance with the reviewer's comment we explain the use of PFA: "PFA is good for preserving cell morphology, and is especially suited to the visualization of membrane bound proteins. The organic solvents, such as methanol, have a dehydrating effect on the cell and remove a lot of small, soluble molecules and lipids in the fixation process, making them unsuitable for use in certain scenarios."

7. **Skin fibroblasts" are mentioned in the abstract but not in the materials.**

   Skin fibroblasts were kindly gifted from Charles University, Faculty of Medicine in Hradec Králové.

8. **The way antibodies are listed in the table is not particularly helpful. It might be better to list their characteristics explicitly, e.g. Polyclonal Rabbit anti-Mouse IgG2a against gamma tubulin.**

   As suggested by the reviewer, we have changed the characteristics of antibodies.

   Monoclonal Anti-Acetylated Tubulin antibody produced in mouse
   Polyclonal Rabbit anti-Mouse IgG2a
   Anti-Mouse IgG (whole molecule) F(ab')2 fragment–Cy3 antibody produced in sheep
   AffiniPure F(ab')$_2$ Fragment Goat Anti-Rabbit IgG

9. **This standardization would also improve the paragraph beginning at line 271. In that paragraph, it is unclear why it is specified that the secondary abs were diluted separately and then incubated concurrently.**

   As suggested by the reviewer we change the paragraph: "In contrast with other protocols, both primary antibodies Monoclonal Anti-Acetylated Tubulin antibody produced in mouse and Anti-γ-Tubulin antibody produced in rabbit were incubated concurrently for 60 minutes using a 1:800 and 1:300 dilution in 1x PBS respectively. In addition, the secondary antibodies, Anti-Mouse IgG (whole molecule) F(ab')2 fragment–Cy3 antibody produced in sheep and Alexa Fluor488 AffiniPure F(ab')$_2$ Fragment Goat Anti-Rabbit IgG were diluted in a 1:300 ratio in 1x PBS. Both were incubated concurrently for 45 minutes."

10. **DAPI dilution is not present.**

    The dilution of DAPI – 1:5,000 has been registered.

11. **Fig 1 - Should include explanation for other green dots present in images. Discuss difference in length of cilium in C.**

    The additional green dots represent background. The length of primary cilium in figure C was measured by ImageJ and we didn't detect any significant changes in length of primary cilia as this primarily a methods paper, further discussions on our results can be seen elsewhere (Filipová A et al. Ionizing radiation increases primary cilia incidence and induces multiciliation in C2C12 myoblasts. Cell Biol Int. 2015, 39(8):943-53. doi: 10.1002/cbin.10462).

12. **Isotype controls and secondary antibody only controls are not included.**

    This is correct. Neither of these were included because this is a methods paper and therefore does not discuss in depth research into primary cilia where such inclusions are a strict requirement for experimental control and publication of results.

13. **It is not clear to me that the addition of gelatin to the coverslips is a critical step. Adherence of the cells to the coverslips could be achieved by coating with other options as well, e.g. poly-lysine. If it is necessary for PC staining, the reason why should be more explicitly stated so that it is not deemed unnecessary by readers with other IF experience.**

Coating cover slips with gelatin is much easier and cheaper than coating with poly-lysine.

14. **The bold and underlined statements in 3.1-3.6 should include an explanation as to why those steps are so critical. Adding a few words to each would add a lot.**

    These are addendums and/or tips into each step of the protocol and as such are self-explanatory.

15. **Cy3 is a relatively poor fluorophore compared to newer options. It would be good to point out that other combinations of secondary antibodies could be used as long as species are compatible with the two primary abs used.**

    As suggested by the reviewer, we pointed out, that other combinations of secondary antibodies could be used as long as species are compatible with the two primary antibodies used.

16. **It is unclear if fixation, blocking, washes, ab incubations, etc. are performed on a shaker or without shaking.**

    All mentioned steps are performed without shaking, otherwise it would have been stated for convenience sake.

17. **To avoid using such a high volume of antibody solution for overnight incubations, use of a humidity chamber could be recommended with the same 150 μL volume of ab solution on coverslips.**

    In accordance with the reviewer's comment we wrote the note that humidity chamber can be used.

18. **Reference 2 is nonstandard.**

    Reference 2 was changed: 2. Primary cilia. Elsevier, Acad. Press. Amsterdam. (2009).

19. **The section of the introduction describing types of signaling dependent on PC-based receptors should be expanded.**

    As suggested by the reviewer, the paragraph was expanded as follows: "Depending on the cell type, the primary cilia express different types of receptors, ion channels and active signaling pathways. The most important signaling receptors affecting *proliferation and survival are EGFR, PDGFR and FGFR. Some of the signaling pathways that may affect the function of one or more organs include Hedgehog, Notch, and Wnt. Thanks to these receptors and signaling pathways, the primary cilia also performs a chemosensory function. This function allows primary cilia to detect specific ligands for Notch, hormones and biologically active substances such as serotonin or somatostatin. Other specific functions exhibited by primary cilia of different length include reaction to changes in temperature, gravity and osmolality."*

20. **A brief aside that expands on example methods of inducing ciliation might be helpful.**

    It is a good suggestion; however, this protocol is solely dedicated toward the detection of primary cilia by immunofluorescent staining.

21. **More references should be cited that describe inducing ciliation in various cell types.**

    As mentioned in the previous point, this protocol is solely dedicated towards the detection of primary cilia by immunofluorescence. Therefore, such suggestion falls beyond the intended scope of this communication.

22. **Pixelation in the figures distracts from visualization of cilia.**
    The format of the images has been changed accordingly.

23. **Repetition in the figure legends is distracting. Consider grouping similar images together into fewer figures.**
    Figure legends have been modified as suggested.

24. **The purpose of the paragraph at line 271 is unclear.**
    This has been addressed accordingly.

25. **Other antibody-mediated methods for detection of primary cilia should at least be mentioned (e.g. Arl13b).**

    We included only the detection of the axoneme and basal body of the primary cilia as this has been implemented as the go-to method. While the use of alternative means is a valid suggestion, care must be taken into which proteins are chosen for such purpose. For example, Ar113b is commonly related to the study of neuronal development and its relationship with pathologies such as Joubert syndrome. Unless the researcher is specifically addressing this subject the use of alternative means of detection might be overkill or produce misleading results.

Dear referee, thank you the time and effort set into the review of our manuscript. It surely improved its quality and impact.

RESPONSE TO REVIEWER _3

The manuscript titled 'Easy detection of primary cilia by immunofluorescence' described immunofluorescence-staining method for the detection of primary cilia in culture cell lines. The manuscript lacks novelty and the protocols presented does not involved any specialized and unique technique. In other word, it's just a common immunofluorescence method that has long been widely applied by the scientist in the field.

Moreover, there were two previous publications; in fact, these two groups provided more detailed and informative methods of visualizing primary cilia by immunofluorescence staining. One group even showed the effect of different fixation methods. Please see the links.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3690948/

https://ciliajournal.biomedcentral.com/articles/10.1186/s13630-017-0045-9

Given the points above. I do not see obvious or strong value of suggesting the publication of the manuscript in the current form.

The reviewer makes a valid point in this regard and, as matter of fact, our protocol also considers these publications. Having said that, the present protocol aims to be straightforward and easily accessible for all researchers, specially those at the beggining of their career when, more often than not, struggle to make heads or tails of the methodology included within a research communication, which is frequently scarce and/or vague.