

Co-staining of NLRX1 with Mitochondria in Glioma Cell Lines --Manuscript Draft--

Article Type:	Invited Encyclopedia of Experiments
Manuscript Number:	JoVE72432R1
Full Title:	Co-staining of NLRX1 with Mitochondria in Glioma Cell Lines
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Additional Information:	
Question	Response

PROTOCOL TITLE:

Co-staining of NLRX1 with Mitochondria in Glioma Cell Lines

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KEYWORDS:

Glioma; Innate Immunity; Mitochondria; NLRs; NLRX1

PROTOCOL OVERVIEW:

The Nucleotide-binding domain, Leucine-rich repeat containing protein (NLR) family of intracellular proteins plays a critical role in innate immunity¹. NLRX1 is a member of the NLR family expressed in mitochondria and cytoplasm². NLRX1 function positively correlates with its subcellular localization. In mitochondria, NLRX1 negatively regulates antiviral signaling by directly interacting with the mitochondrial antiviral-signaling protein (MAVS) on the outer membrane³. This interaction negatively regulates the production of Interleukin 6 (IL-6) and type 1 Interferon (IFN-1), and possibly inhibits NLRP3 inflammasome formation. In the mitochondrial matrix, NLRX1 interacts with ubiquinol-cytochrome c reductase core protein 2 (UQCRC2), a subunit of complex III of the electron transport chain. This interaction induces the generation of reactive oxygen species (ROS), which in turn activates the c-Jun N-terminal kinase (JNK) pathway and results in apoptosis⁴. NLRX1 can be associated with the mitochondrial immune signaling complex (MISC) and mitochondrial Tu translation elongation factor (TUFM) to promote autophagy⁵. Thus, NLRX1 is an essential regulator of mitochondrial signaling. Mitochondria are functionally versatile organelles. In addition to their conventional role of fulfilling the cell's energy requirements, mitochondria also actively regulate innate immune responses⁶. Innate immune proteins have a significant part in regulating mitochondrial signaling. Therefore, understanding mitochondrial signaling and its crosstalk with innate immune signaling is essential to interpreting cell physiology. In this protocol, we present a method to perform mitochondrial staining in live cells using MitoTracker and a method to perform colocalized staining of mitochondria and innate immune protein NLRX1 in fixed cells (**Figure 1**). In addition to MitoTracker dye, several approaches are available for mitochondrial visualization^{7,8}. For example, antibodies targeting mitochondrial proteins such as TOM20 or Cytochrome c can be used to label mitochondria. In addition, membrane potential-dependent dyes, including TMRM, TMRE, and JC-1, are commonly employed to assess mitochondrial metabolic status and membrane potential. However, MitoTracker dyes offer several advantages, including ease of use, high fluorescence intensity, and

the ability to retain mitochondrial labeling following aldehyde fixation⁸. Owing to these advantages, MitoTracker represents a versatile and widely applicable tool for mitochondrial visualization. The protocol would be helpful for the study of mitochondrial signaling and its crosstalk with innate immune signaling. This protocol can also be utilized to study colocalization by immunostaining other innate immune proteins with mitochondria.

PROTOCOL:

The reagents and the equipment used are listed in the **Table of Materials**.

1. Preparation

1.1. Fixative agent: Prepare 4% paraformaldehyde (PFA) from stock reagent in 1x phosphate-buffered saline (PBS).

1.2. Prepare permeabilization buffer by adding 0.1% Triton X-100 to 1x PBS.

1.3. To prepare the blocking buffer, add 5% fetal bovine serum (FBS) to the permeabilization buffer.

1.4. Prepare anti-NLRX1 rabbit primary antibody dilution (1:1000) in blocking buffer.

1.5. Prepare anti-rabbit fluorophore-conjugated secondary antibody dilutions (1:1000) in blocking buffer.

2. Mitochondrial staining in live cells

2.1. Prepare 1 mM Mito Tracker stock solution in dimethyl sulfoxide (DMSO). Mix the solution quickly by vortexing. Store the stock solution at -20 °C in dark conditions.

2.2. Seed the glioma (LN-229, LN-18, U87-MG) and astrocyte cells (SVG) in a chamber slide at 50,000 cells/well density in a complete growth medium (DMEM + 10% FBS) with 1% antibiotic-antimycotic solution (Penicillin, Streptomycin, Amphotericin B).

NOTE: Cell density can vary depending on the cell type.

2.3. When the cell density reaches almost 60%–70% (cells are abundant and actively dividing, but they still have visible empty spaces between colonies), remove the medium from the cells. Add 1 mL of serum-free DMEM without antibiotics to the cells. Now, add the Mito Tracker solution to the medium to achieve a final concentration of 25 nM. Incubate the cells in a CO₂ incubator in dark conditions for 30 min.

2.4. After the incubation, remove the media (containing Mito Tracker) from the cells and wash the cells 3x with serum-free DMEM. Add fresh medium [complete growth medium (DMEM + 10% FBS) with antibiotic-antimycotic solution] to the cells and perform live cell imaging.

89

90 **3. Colocalized staining of NLRX1 with mitochondria**

91

92 3.1. Follow steps 2.1–2.3, discard the medium from the cells, and wash the cells with 1x PBS
93 to remove the remaining medium.

94

95 3.2. Add 1 mL of 4% PFA to the cells and incubate for 10 min at room temperature (RT).

96

97 CAUTION: PFA is harmful upon direct contact with skin or eyes.

98

99 3.3. After fixation, remove PFA from the cells and wash them 2x for 7 min or 3x for 5 min with
100 1x PBS at RT.

101

102 NOTE: Wash the cells gently; use a slow rocker. After fixation, cells can be placed at 4 °C in 1x PBS
103 for up to 1 week.

104

105 3.4. Add 1 mL of permeabilization buffer to the cells and incubate for 15 min at RT.

106

107 3.5. Discard the permeabilization buffer, add 1 mL of blocking buffer to the cells, and place
108 them at 4 °C for 1.5 h in a humidified chamber.

109

110 NOTE: The blocking duration can be increased if a non-specific background signal is observed
111 after staining.

112

113 3.6. Remove the blocking buffer, add ~500 µL of the primary antibody solution to the cells,
114 and incubate overnight (13–15 h) at 4 °C in a humidified chamber.

115

116 NOTE: Primary antibody incubation time can vary depending on the antibody and the cells used
117 in the experiment. Set aside one well as a primary antibody control; add blocking buffer only to
118 this well without any primary antibody.

119

120 3.7. The next day, discard the primary antibody solution and wash the cells with 1x PBS as
121 mentioned in step 3.3.

122

123 3.8. After washing, add ~500 µL of the secondary antibody solution to the cells and incubate
124 for 1 h at 37 °C in dark conditions in a humidified chamber.

125

126 NOTE: Add secondary antibody in the primary antibody control cells, too.

127

128 3.9. Wash the cells with 1x PBS as mentioned in step 3.3.

129

130 3.10. Remove excess PBS using tissue paper or laboratory wipes. Add 40 µL of mounting medium
131 to the cells, then place a clean coverslip on top. The mounting media also contains DAPI, which
132 stains cell nuclei. Leave the slides at RT for 1 h and store them at 4 °C.

NOTE: Do not let the slide dry to avoid salt deposition, which can result in an elevated non-specific background staining signal.

REPRESENTATIVE RESULTS:

For MitoTracker green, the $\lambda_{\text{excitation}}$ maximum is ~ 490 nm, and the $\lambda_{\text{emission}}$ maximum is ~ 516 nm⁸. The green puncta represent MitoTracker staining as MitoTracker fluoresces in the green channel, and the red puncta represent NLRX1 as it was stained with red fluorophore-conjugated secondary antibody (**Figure 2** and **Figure 3**). Therefore, the green signals indicate mitochondria, and the red signal indicates the NLRX1 protein. In the overlaid images, the yellow signal indicates colocalization of MitoTracker and NLRX1 staining and thus indicates NLRX1 in mitochondria⁹. Here, cells without anti-NLRX1 primary antibody treatment were taken as the negative control for the primary antibody, and cells without MitoTracker treatment were taken as the negative control for MitoTracker. Isotype antibody controls may be utilized as primary antibody controls. Further, the colocalization of NLRX1 with MitoTracker was quantified by calculating Pearson's correlation coefficient using ImageJ (**Figure 3B**).

FIGURES:

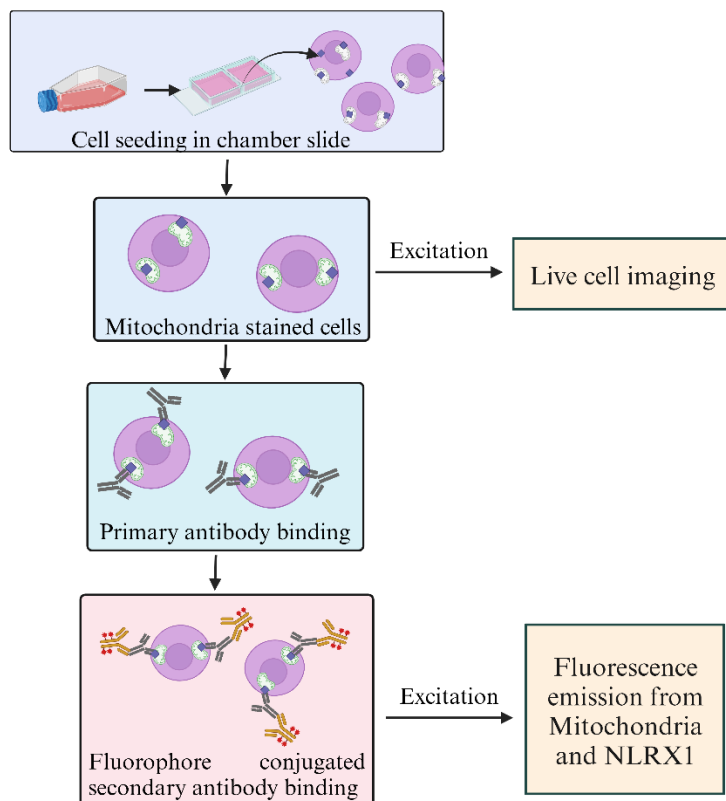


Figure 1

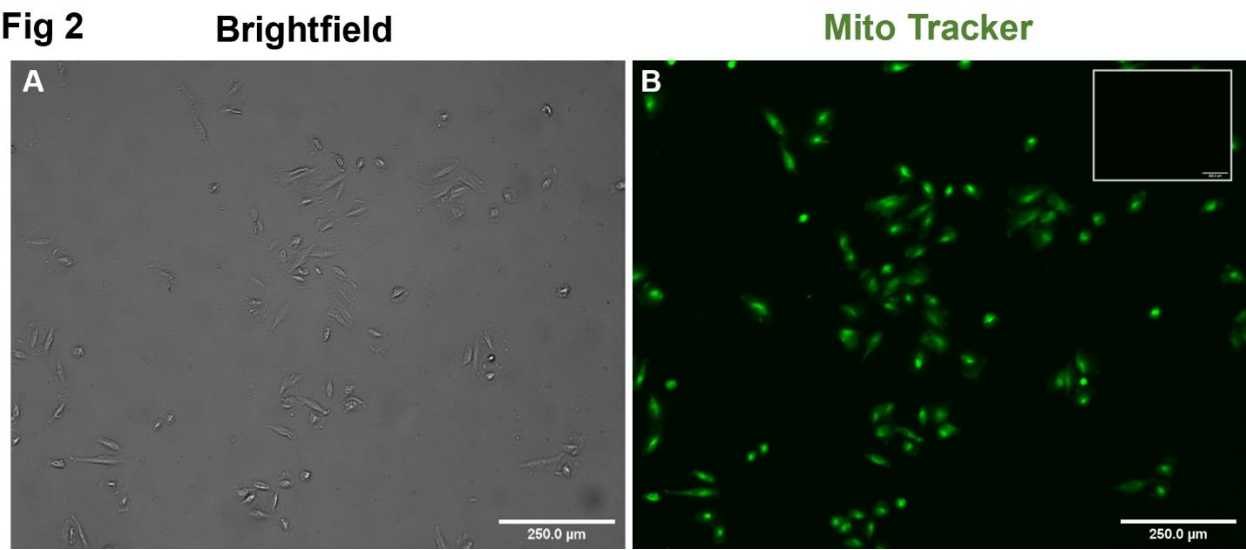


Figure 2

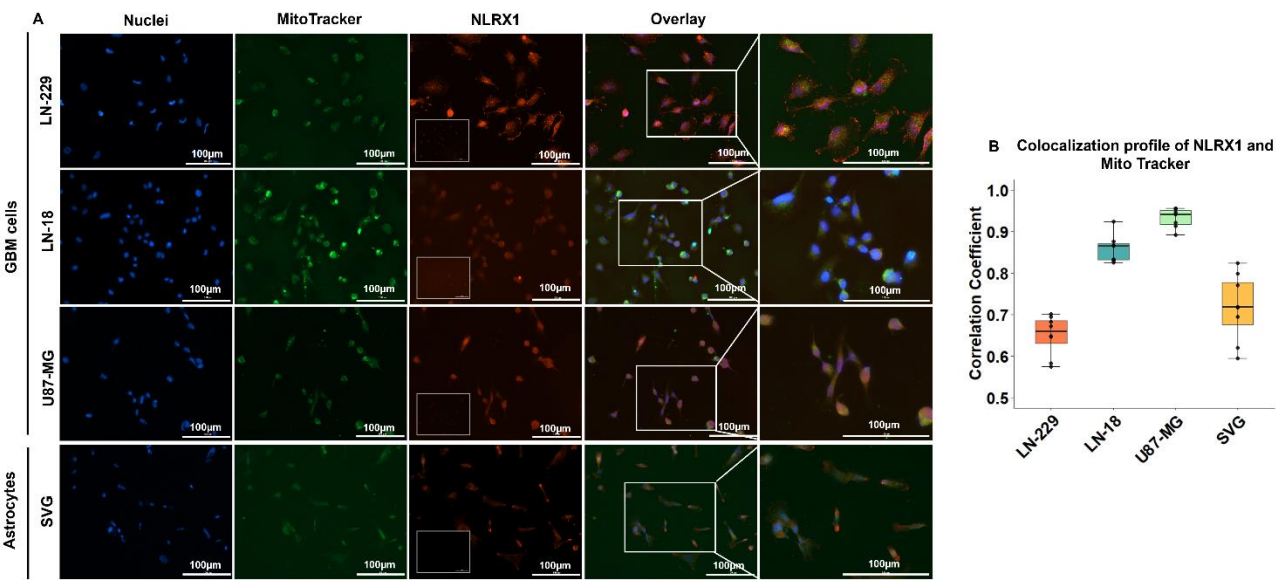


Figure 3

FIGURE LEGENDS:

Figure 1: Schematic of the protocol. Glioma (LN-229, LN-18, U87-MG) and astrocyte (SVG) cells were seeded in a chamber slide. At 70%–80% cell density, the media was removed from the cells and treated with MitoTracker. After MitoTracker incubation, cells were fixed, permeabilized, blocked, and then treated with a primary antibody specific for NLRX1. After primary antibody

incubation, a secondary antibody was added to the cells. The cells were mounted in DAPI-containing mounting media and imaged with a fluorescence microscope.

Figure 2: Mito Tracker staining in live cells. Glioma (LN-229) cells were treated with MitoTracker green (25 nM). (A) Brightfield image; (B) Fluorescent image of MitoTracker-treated LN-229 cells. Images were captured at 4x. Scale bar is 250 μ m. The inset represents MitoTracker control.

Figure 3: Colocalization of innate immune protein NLRX1 with MitoTracker. (A) GBM (LN-229, LN-18, U87-MG) and astrocyte (SVG) cells were stained for NLRX1 and Mitochondria. Through immunocytochemistry, nuclei were stained with DAPI (blue), mitochondria were stained with MitoTracker (green), and NLRX1 (red) was stained by NLRX1-specific immunolabeling. Images were captured at 20x and 40x in a fluorescence microscope. Scale bar is 100 μ m. Insets represent NLRX1 Primary antibody control. (B) From the overlay images, colocalization of NLRX1 and MitoTracker was quantified by calculating Pearson's correlation coefficient between NLRX1 expression (red) and MitoTracker expression (green) through the JACoP plugin in ImageJ. These figures have been modified from Meena et al.⁹.

DISCUSSION:

This protocol presents a method for MitoTracker staining in live cells and for colocalized staining of the innate immune protein NLRX1 with MitoTracker in fixed cells⁹. Cells were grown in a complete medium with antibiotics. The complete medium was removed, and serum-free medium was added to the cells before MitoTracker addition, as serum proteins interfere with MitoTracker staining. After the MitoTracker addition, cells were placed in the dark in a CO₂ incubator, as the light could quench the fluorescence coming from the MitoTracker. After completion of the MitoTracker incubation, the cells were washed with medium, then with PBS to remove excess MitoTracker. Next, the cells were fixed with PFA to perform NLRX1 staining, along with MitoTracker. PFA cross-links proteins by forming an insoluble meshwork. After fixation, cells were permeabilized, which creates pores in the cell membrane by disrupting lipid organization at the membrane. This allows blocking buffers and antibodies from entering the cells through the porous cell membrane.

After permeabilization, cells were incubated in blocking buffer containing FBS. Serum proteins bind and block the antigenic sites or antibody binding sites present in the cell. This aims to improve the specificity of the primary antibody binding¹⁰. As all antibody binding sites are blocked by FBS, only primary antibodies with high specificity and binding efficacy can replace the serum protein and bind to specific antigenic sites. Thus, this step is necessary to avoid non-specific primary antibody binding. After primary antibody incubation, a PBS wash was given to remove the unbound antibodies. Next, a secondary antibody conjugated with a fluorophore and specific to the primary antibody was added to the cells. The secondary antibody binds to the primary antibody and, upon excitation, fluoresces. Further, a PBS wash was performed to reduce the risk of non-specific binding of secondary antibodies. After that, cells were mounted in mounting media. Mounting helps protect the sample for a longer period, and DAPI stains the cell nuclei. Additionally, to quantify the NLRX1 colocalization with mitochondria, Pearson's correlation coefficient was calculated between NLRX1 and MitoTracker signal.

TABLE OF MATERIALS:

Name of Material/ Equipment	Company	Catalog Number
10x-PBS	Himedia	ML023-500ML
37% Paraformaldehyde	Himedia	MB059-500ML
Alexa Fluor 594 nm anti-rabbit antibody	Life Technologies	A11012
Antibiotic-antimycotic solution	Himedia	A002-100ml
Anti-NLRX1 Primary Antibody	Novus Biologicals	NBP1-76287
Chamber slide	SPL	30112
Coverslip	Blue Star	
DMEM	Cell Clone	CC3004.05L
DMSO	Cell signalling technology	12611P
FBS	Cell Clone	CCS-500-SA-U
Fluorescence Microscope	BioTek	
Fluoroshield TM with DAPI	Sigma	F6057-20ML
Mito Tracker Green FM	Thermofisher	7514
Triton-X-100	Sigma	1001723790

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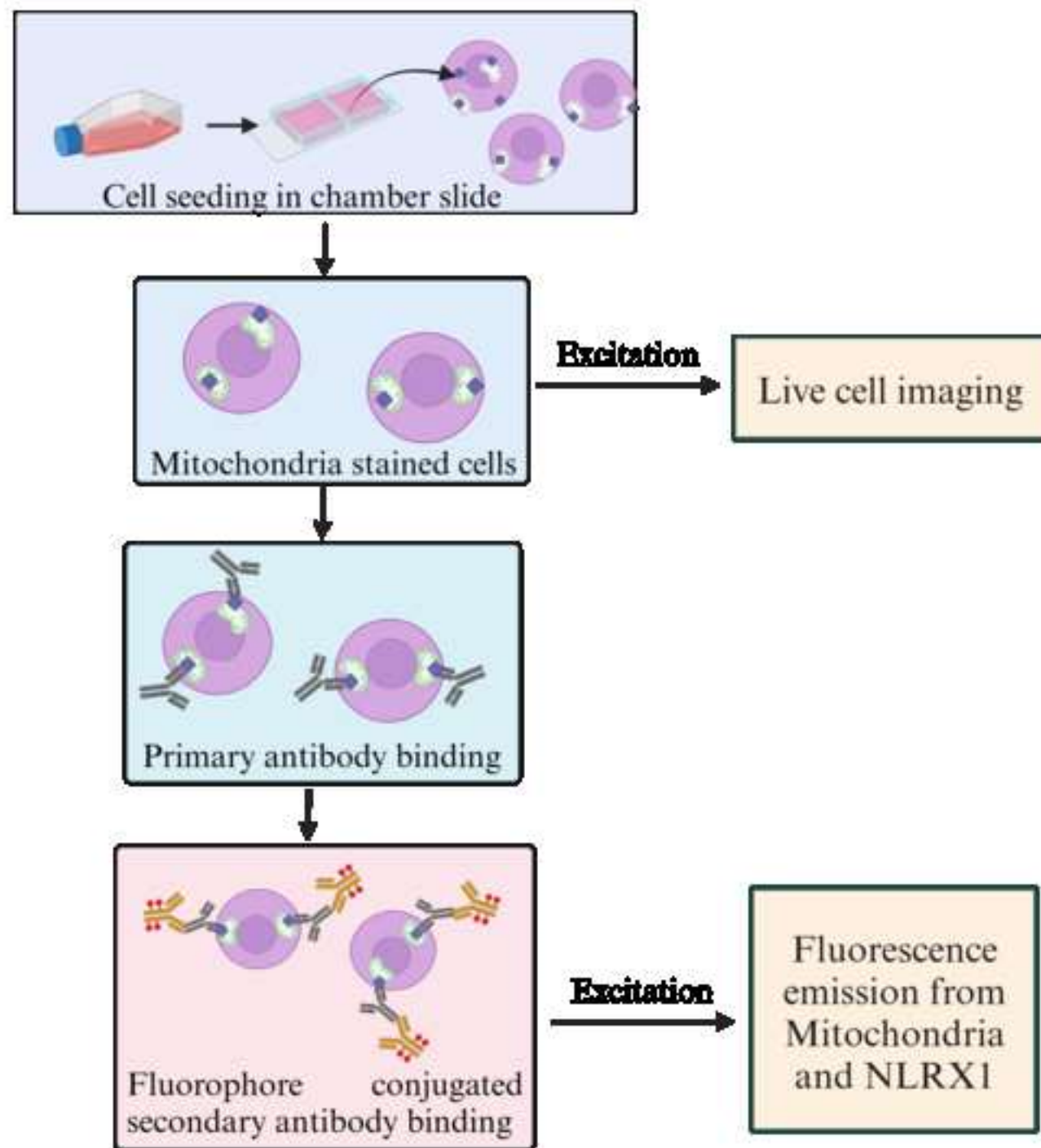


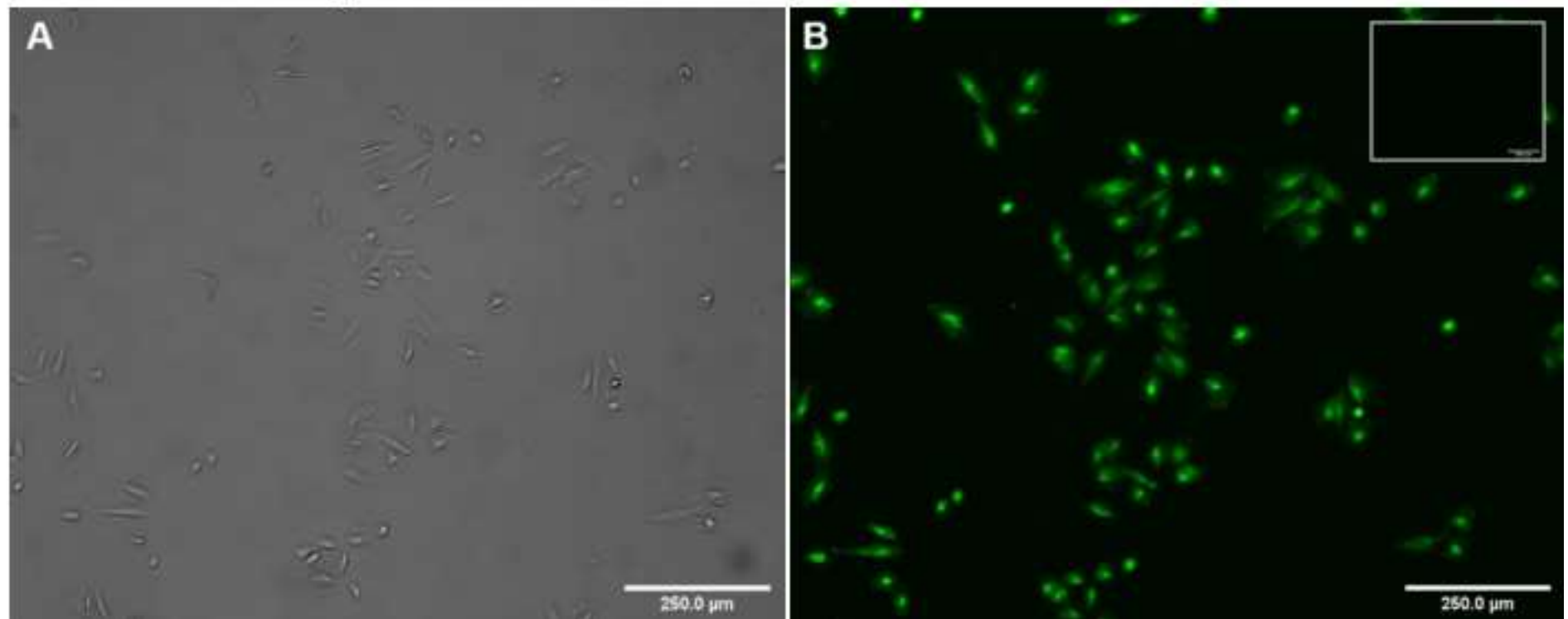
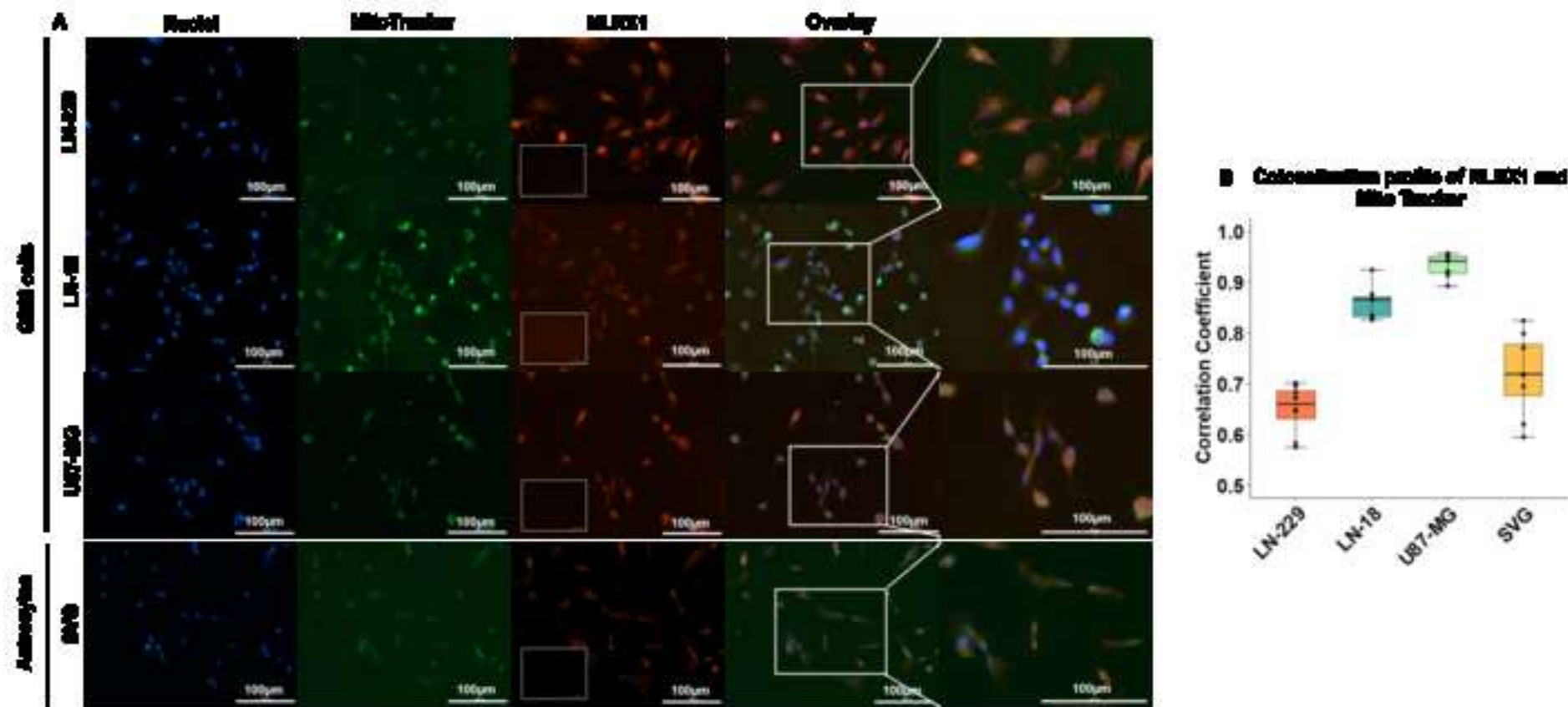
Fig 2**Brightfield****Mito Tracker**

Figure 3



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37% Paraformaldehyde	Himedia	MB059-500ML
Alexa Fluor 594 nm anti-rabbit antibody	Life Technologies	A11012
Antibiotic-antimycotic solution	Himedia	A002-100ml
Anti-NLRX1 Primary Antibody	Novus Biologicals	NBP1-76287
Chamber slide	SPL	30112
Coverslip	Blue Star	
DMEM	Cell Clone	CC3004.05L
DMSO	Cell signalling technology	12611P
FBS	Cell Clone	CCS-500-SA-U
Fluorescence Microscope	BioTek	
FluoroshieldTM with DAPI	Sigma	F6057-20ML
Mito Tracker Green FM	Thermofisher	7514
Triton-X-100	Sigma	1001723790