NextGen Protocols

TITLE: Immunofluorescent Staining for γH2AX in Cells

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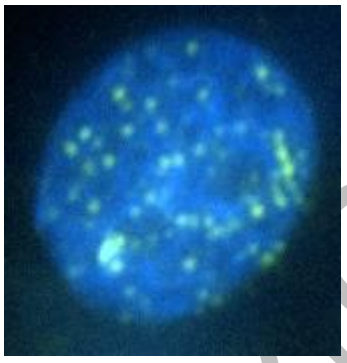
MOTIVATION:
• DNA damage is associated with an increased risk of cancer, aging and disease
• γH2AX is a widely accepted marker for the response to DNA double strand breaks
• This protocol demonstrates a method to stain and quantify the double strand breaks in an *in vitro system.*

EXAMPLE DATA:



γH2AX Positive MDCK Cell irradiated with 50Gy of Gamma Radiation

PROTOCOL:

Step 1: Gelatinizing the Coverslips

Overview
This process prepares the coverslips to be adherent to the cells. All of this work should be performed in BSL2 Biosafety cabinet
Active Time: 5 minutes
Total Time: 1 hour 35 minutes

Materials:
Falcon 12-well Flat Bottom Culture Plate with lid (Corning 353043)
18 mm #1 Circular Coverslips (Electron Microscopy Sciences 72229-01)
Gelatin from Porcine skin (Sigma G1890)

1.1 Mix 5 g of Gelatin with 500 mL of distilled water
1.2 Autoclave the 1% Gelatin solution until sterile
1.3 Pipet 1 mL of 1% gelatin solution to well in 12-well plate
1.4 Place individual coverslip into each well with gelatin (**Video #1**)
1.5 Use pipet to press coverslip to bottom of well (**Video #1**)
1.6 Rotate plate to ensure gelatin covers entire coverslip (**Video #1**)
1.7 Place 12-well plate into 37°C incubator for 1 hour
Tip: Plate can be in incubator for 1-6 hours without effect

Step 2: Plating the Cells

Overview
This step is for seeding the cells prior to beginning the experiment. All of the cell culture in this step should be performed in a BSL2 biosafety cabinet
Active Time: 20 minutes
Total Time: 20 minutes

Materials:
Trypsinizied cell solution
Cell specific media

2.1 Quantify the number of cells in the cell solution
2.2 Centrifuge vial of trypsinized cells at 1000 RPM for 5 minutes at 4°C
2.3 Aspirate trypsin solution from the cells
Tip: Be careful not to aspirate the pellet
2.4 Resuspend the cells to 1x 10^6 Cells/mL
2.5 Remove 12-well plate from incubator and aspirate gelatin
2.6 Plate 1 mL of cell-specific media to each gelatinized well in 12-well plate
2.7 Pipet 0.3 x 10^6 cells to each well
2.8 Rotate plate 360° to ensure complete coverage of area with cells
2.9 Place plate in 37°C incubator overnight

Step 3: Experimentation and Fixation

Overview
This process is for performing the actual experimentation and fixation.
Active Time: 20 minutes
Total Time: 20 minutes

Materials:
4% Paraformaldehyde aqueous solution (Electron Microscope Sciences #157-4)
Sterile Dulbecco's Phosphate Buffered Saline (Life Technologies 14190-144)

3.1 Perform desired experiment on cells
Note: This experiment can be any procedure that does not require the removal of the cells from the plate.

3.2 Upon completion of the experiment, aspirate liquid in wells of 12-well plate

3.3 Pipet 0.5 mL of 4% paraformaldehyde to each well
Note: Paraformaldehyde is extremely toxic and should be used inside a ventilated fume hood.
Note: You cannot fix wells independently of one another, because 4% paraformaldehyde on one side of the slide fixes cells on the other side of the plate

3.4 Incubate wells at room temperature for 10 min

3.5 Removed paraformaldehyde and wash wells twice with PBS
Note: At this point, the cells can be incubated with 1mL PBS and placed in 4°C for two weeks or until further use

Step 4: Immunofluorescent Staining

Overview
This process is for performing the actual permeabilisation, staining and mounting the slides
Active Time: 40 minutes
Total Time: 3 hours + 1 overnight

Materials:
Fine forceps
Triton x-100 (Sigma-Aldrich X100)
Tris Buffered Saline (BioRad 170-6435)
Bovine Serum Albumin (Sigma-Aldrich A3059)
Anti-phospho-Histone H2A.X(Ser139) Antibody, clone JBW301 (Millipore 05-636)
Goat anti-mouse IgG-Fitc (Santa Cruz sc-2010)
26G x ½ Regular Bevel Needles (Becton Dickinson 305111)
1 mL Tuberculin Slip Tip Syringe (Becton Dickinson 309569)
75X25 mm Single End Frosted Slides (VWR 16004-368)
Prolong Gold Anitfade with DAPI (Life Technologies P36935)

4.1 Dilute 20 µL of Triton X-100 in 10 mL of TBS to produce permeabilization buffer (0.2% Triton X-100 in TBS)

4.2 Dissolve 0.1g of BSA in10 mL TBS to produce blocking buffer (1%BSA in TBS)
Note: This is enough blocking buffer to performs stains on 8 wells. Scale volume accordingly.

4.3 Aspirate PBS from wells of the 12 well plate

4.4 Incubate each well with 0.5mL of Permeabilization Buffer for 10 min at room temperature

4.5 Aspirate Permeabilization Buffer

4.6 Incubate each well with 0.5mL of Blocking Buffer for 1 hour at room temperature

4.7 Dilute 6 µL of anti-yH2AX Antibody in 3 mL of Blocking Buffer (1:500 Dilution)

4.8 Aspirate Blocking Buffer

4.9 Pipet 500 µL of Primary Antibody solution to each well in plate

4.10 Incubate each plate overnight at 4°C with gentle rocking

4.11 Store blocking buffer at 4°C

Next Day:

4.12 Aspirate Primary Cell Solutions

4.13 Wash all wells with 0.5mL of TBS to each well twice aspirating after each wash

4.14 Dilute 6µL Goat- anti-mouse-Fitc in blocking buffer (1:500 Dilution)

4.15 Wash all wells with 0.5 mL of TBS twice aspirating after each wash
4.16 Bend Syringe needle to a 90 angle on counter with tip facing down **(Video #2)**
4.17 Pipet 5 uL of ProLong Antifade onto glass slide
4.18 Using Bent Syringe and forceps, pickup coverslip and flip onto slide **(Video #3)**
4.19 Image slide as soon as possible
4.20 Store slides at -20C until imaged

Step 5: Imaging and Quantification

Overview
This process is to learn how to image and quantify yH2AX pictures

Active Time: 3 hours
Total Time: 3 hours

Materials:
Fluorescent microscope

5.1 Place slide on microscope stage and visualize at 10x under DAPI filter
5.2 Switch to 60x magnification and focus
5.3 Capture images of the slide first under DAPI filter and subsequently under Fitc filter
5.4 Using your image software program, create a Dapi/Fitc merged image
5.5 Take 5-10 images per slide throughout the entire slide area
Note: To be properly unbiased, search under DAPI filter
5.6 Upon capturing images for every slide, have a colleague blind you to the name of every image
5.7 Count the number of Dapi positive cells in that image
5.8 Count the number of cells with >5 yH2AX foci in that image
5.9 Repeat for remaining images.
5.10 Unblind yourself to the images names.
5.11 Sum the total number of DAPI positive cells and cells with >5 yH2AX foci for each original slide
5.12 Finally quantify the percentage yH2AX positive using the equation below:

$$\frac{Total\ \#\ of\ cells\ with > 5\ yH2AX\ foci}{Total\ \#\ of\ DAPI\ Positive\ Cells} * 100\%$$
$$= Percetange\ yH2AX\ Positive\ Cells\ Per\ Condition$$