Gamma-H2AX protocol for attached cells.

Prepatation
Grow cells on 22x22 mm coverslips.
Do the fixation and staining procedures in Petri dishes. Make sure cells do not get dry between the washing steps.

1) **Fixation**

- Remove media. Fix cells 10 min in 1 ml 70% Ethanol.
Alternative: 3% Paraformaldehyde, 2% Sucrose in PBS. Room temperature! Remove liquid.

2) **Permeabilization**

- Add 1 ml 0.2% Triton X per sample. Permeabilize cells for 5 min in room temperature
- Wash 3 times with 2.5 ml PBS

3) **Immunostaining**

- Add 100 µl primary antibody*/sample: concentration 1:800 in PBS supplemented with albumin*. Cover with parafilm square to keep moist.
- Incubate for 30 min in 37 degrees in wet chamber.
- Wash 3 times with 2.5 ml PBS

From now on : **Work in the dark!**
Add 100 µl secondary antibody*/sample: Concentration 1:200 in PBS supplemented with albumin*. Cover with parafilm square.
- Incubate for 30 min in 37 degrees (wet chamber)
- Wash 3 times with 2.5 ml PBS

4) **Counterstaining (DAPI)**

- Stain with 100 µl DAPI (0.000025% in PBS) per sample for 10 min at room temperature. Parafilm/wet chamber.
- Wash 3 times with 2.5 ml PBS

5) **Mounting**

- Mount coverslips in Vectashield. Seal with Nail Varnish or Glue

6) **Scoring**

- Take images, analyse with ImageJ

Chemicals:

Primary antibody: Upstate Cell Singnalling Solution NY, anti-phospho-Histone H2AX (Ser139) Mouse monoclonal, cat no. 05-636

Secondary antibody: Sigma Steinheim Germany, anti-mouse IgG FITC, cat-no: F0257

Albumin: 2% Bovine Serum Fraction V albumin

Solution volumes for gamma-H2AX assay

Solution	**Concentration**	**Volume needed per sample**
Triton X	1 + 49 (10%Triton X in PBS)	1 ml
PBS-BSA	4+1 (4 PBS + 1BSA)	enough for 1' & 2' ab
1' ab	1:1000 (in PBS-BSA)	150 µl
2' ab	1:400 (in PBS-BSA)	150 µl
DAPI	1:100 (in PBS)	150 µl

Trouble shooting

High unspecific binding: use PBST (PBS +0,05% Tween 20) for washing.
Increase the washing steps and waiting time between each wash.
Add a blocking step: minimum 30min blocking in 2%BSA in PBS.

High background: After DAPI, wash with distilled water.

Uneven nucleus: make sure the cells did not get dry under the staining procedure.