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Tumor Microenvironment

Study Protocols

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Preface

It is universally accepted that the tumor microenvironment is extremely relevant to both the study of cancer biology and the search for improved therapies. However, it is also accepted that the study of biological mechanism in conditions which accurately mimic this environment is both technically challenging and highly specialized. At a recent tumor microenvironment meeting held in Mykonos, Greece, we decided that it would aid the field in general to publish detailed protocols, far exceeding the level of detail usually reported in papers. We hope that these prove useful and that we as a community can continue to share our collective expertise.

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Chapter 1
Staining Against Phospho-H2AX (γ-H2AX) as a Marker for DNA Damage and Genomic Instability in Cancer Tissues and Cells

Anika Nagelkerke and Paul N. Span

Abstract Phospho-H2AX or γ-H2AX- is a marker of DNA double-stranded breaks and can therefore be used to monitor DNA repair after, for example, irradiation. In addition, positive staining for phospho-H2AX may indicate genomic instability and telomere dysfunction in tumour cells and tissues. Here, we provide a protocol to perform immunostaining for phospho-H2AX on cells, cryosections and formalin-fixed, paraffin-embedded tissues. Crucial steps in the protocol and troubleshooting suggestions are indicated. We also provide suggestions on how to combine staining against γ-H2AX with stainings against components of the tumour microenvironment, such as hypoxia and blood vessels.

Keywords Histone 2A • DNA damage repair • Genomic instability • Telomere dysfunction • Immunohistochemistry • Immunofluorescence • Immunocytochemistry

1.1 Introduction

This protocol provides a procedure to stain cells for phospho-H2AX. H2AX is a modified version of the histone H2A. In the nucleus of cells, the DNA is wrapped around these and other histones, to ensure proper organisation of the DNA.

When DNA damage occurs, a complex cellular response is activated. This DNA damage response (DDR) involves the detection of the damaged site, the amplification of the signal through a cascade of protein kinases and the activation of a series of downstream effectors that promote cell cycle arrest, DNA repair or activation of apoptotic

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pathways [1]. An early event in the DDR is the phosphorylation of the histone H2AX at serine 139. This so-called γ-H2AX modification is dependent on the action of members from the phosphatidylinositol 3-kinase (PI3K)-like family of kinases, which includes ataxia telangiectasia-mutated (ATM), AT-related (ATR) and DNA-dependent protein kinase (DNA-PK) [2]. Depending on the type of lesions induced, different DNA repair mechanisms are activated. In eukaryotic cells, damaged bases and nucleotides are repaired by base excision repair (BER) and nucleotide excision repair (NER) pathways, respectively, while DSBs are repaired by two major mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ) [1].

Phospho-H2AX is a very robust marker of DNA double-stranded breaks, which can be stained for. This will visualise phospho-H2AX foci—bright dots—in the nucleus. These foci can be quantified by counting the number of positive cells or by counting the number of foci per nucleus. We have used this staining in the past to follow DNA damage repair kinetics, using phospho-H2AX as a marker [3]. We cultured cancer cells on coverslips, irradiated them with 2 Gy and fixed the cells after 0, 1, 3, 24 and 48 h. We stained for phospho-H2AX and analysed the number of positive cells. This allows quantification of the repair of DNA double-stranded breaks over time. As we were setting up this staining, we observed that cancer cells could exhibit unusual behaviour when it comes to the presence of phospho-H2AX in their nuclei. We noticed that even without being irradiated, cancer cells could display phospho-H2AX foci, a feature that is absent in normal, healthy cells [4, 5]. This positivity has been related to genomic instability and potentially to telomere erosion and dysfunction [6]. We therefore believe that this protocol is not only useful to monitor repair of DNA double-stranded breaks after DNA damage in a number of cell types (not limited to cancer cells), but can also provide a tool to study genomic instability. Combining staining against H2AX with stainings against microenvironmental parameters can provide spatial information on where DNA damage or genomic instability is most prevalent within the tumour microenvironment, for example relative to regions of hypoxia, blood vessels, necrotic tissue, etc.

This protocol contains details for fixed cells, cryo- and formalin-fixed paraffin-embedded (FFPE) sections. We also give an example on how a staining for multiple markers in combination with H2AX can be performed on cryosections, enabling analysis of phospho-H2AX expression in connection with the tumour microenvironment (hypoxic regions and proximity to blood vessels). Examples of the end results of our stainings are provided in Figs. 1.1 and 1.2.

1.2 Protocol

1.2.1 Materials Needed

1.2.1.1 General

- Staining dish and rack, and staining tray (see Fig. 1.3a, b)
- Tris-buffered saline (TBS, 10 mM Tris base, 150 mM NaCl, pH 7.4)

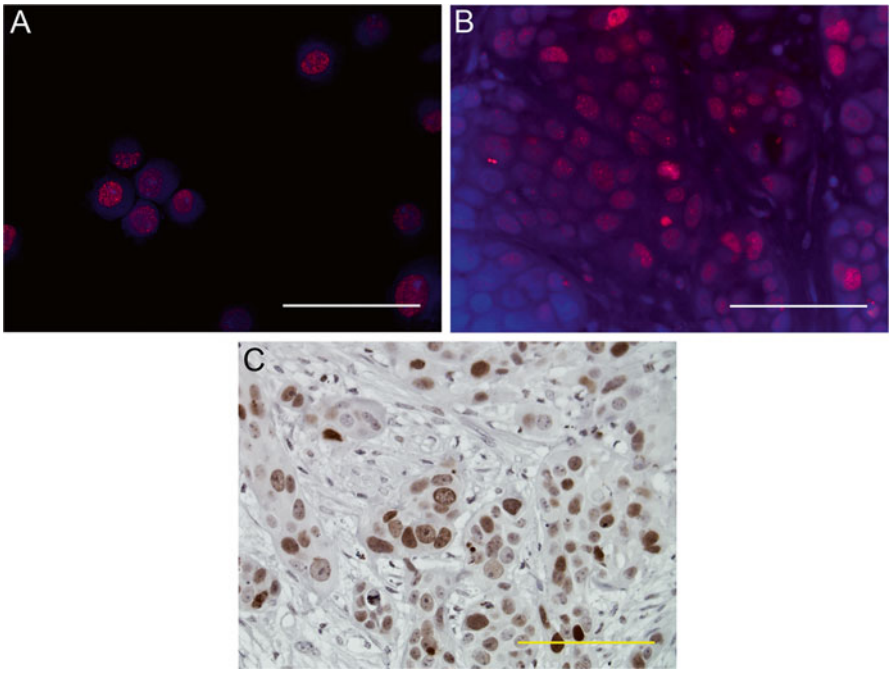

Fig. 1.1 Examples of phospho-H2AX stainings on fixed cells (**a**), frozen sections (**b**) and FFPE sections (**c**). The MDA-MB-231 breast cancer cells in (**a**) were irradiated with 2 Gy and fixed after 1 h. The SSCNij3 head and neck squamous cell carcinoma xenografts of (**b**) and (**c**) were irradiated with 10 Gy and harvested after 24 h. Scale bars equal 100 μm

- Bovine serum albumin (BSA, A7906, Sigma Aldrich, St. Louis, MO, USA)
- Triton X-100 (T8787, Sigma Aldrich, St. Louis, MO, USA)
- Rabbit-anti-phospho-H2AX (#2577, Cell Signaling Technology, Danvers, MA, USA)
- Super PAP-pen (00-8899, Life Technologies, Carlsbad, CA, USA)

1.2.1.2 For Fixed Cells and Cryosections

- Cy3-conjugated AffiniPure Fab fragment donkey-anti-rabbit (711-167-003, Jackson ImmunoResearch, West Grove, PA, USA) or equivalent
- Hoechst 33345 (B2261, Sigma Aldrich, St. Louis, MO, USA)
- Fluoromount W (21634.01, Serva, Heidelberg, Germany)

Optional:

- 9 F1 (undiluted supernatant from 9 F1 cells, which produce a monoclonal antibody to mouse endothelium)

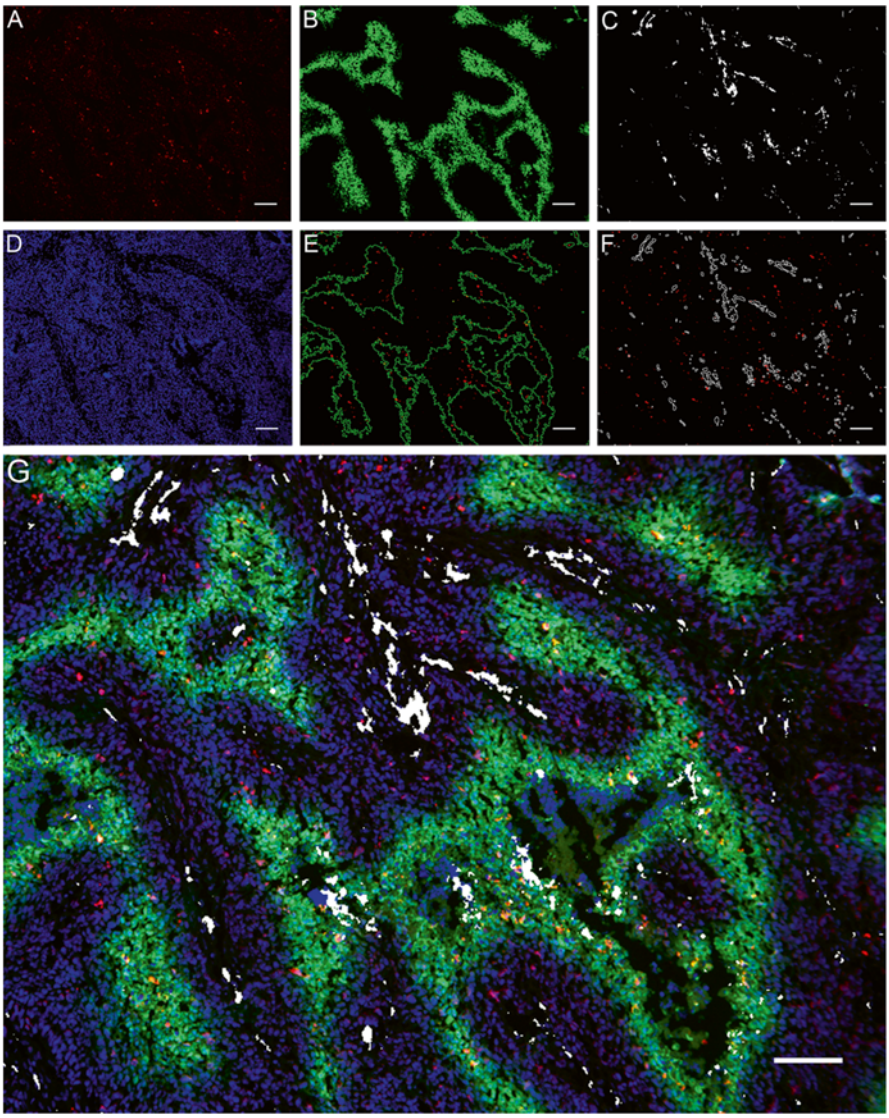

Fig. 1.2 Example of a phospho-H2AX staining (**a**) on a cryosection of a C38 colon carcinoma xenograft with endogenous expression of phospho-H2AX. Phospho-H2AX was combined with a staining against hypoxia with pimonidazole (**b**), blood vessels (**c**) and nuclei (**d**). (**e**) and (**f**) represent how phospho-H2AX is localised with respect to hypoxia and vasculature. A composite image is shown in (**g**). Scale bars equal 100 μm

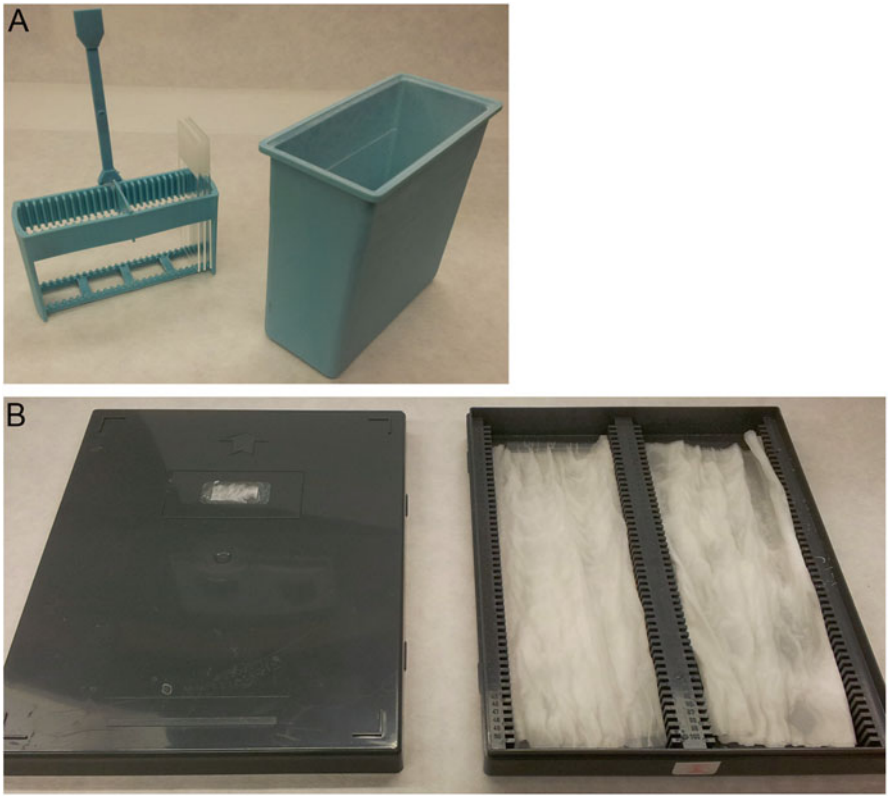

Fig. 1.3 Example of a staining dish and rack (**a**). All rinse steps are performed in these dishes. Example of a staining tray (**b**). All incubation steps are performed in this tray. Wet tissues are added to bottom of the tray to prevent evaporation

- Rabbit-anti-pimonidazole (a kind gift from J.A. Raleigh, also commercially available from Hypoxyprobe, Inc., Burlington, MA, USA)
- Alexa 647-conjugated chicken-anti-rat (A21472, Life Technologies)
- Alexa 488-conjugated donkey-anti-rabbit (A21206, Life Technologies)

1.2.1.3 For FFPE Sections

- Histosafe (Adamas Instruments BV, Leersum, The Netherlands)
- 70 %, 90 %, 96 %, 100 % EtOH
- Target retrieval solution pH 6.0 (S2369, DAKO, Copenhagen, Denmark)
- H_2O_2 (30 %, 76051800, Boom, Meppel, The Netherlands)
- Methanol
- Normal donkey serum (017-000-001, Jackson ImmunoResearch)

- Biotin SP-conjugated Affinipure Fab fragment donkey-anti-rabbit (711-067-003, Jackson ImmunoResearch)
- Vectastain ABC kit elite (PK-6100, Vector, Burlingame, CA, USA)
- DAB, peroxidase substrate kit (SK-4100, Vector)
- Hematoxylin (S3301, DAKO)
- Mounting medium (KP7275, Klinipath, Duiven, The Netherlands)

1.2.2 Procedure

1.2.2.1 Fixed Cells and Cryosections

To stain for phospho-H2AX in cells, we fix them in ice-cold (−20 °C) MetOH for 10 min at 4 °C. Cryosections can be fixed in cold (4 °C) acetone for 10 min at 4 °C. Do not fix cells cultured on polystyrene dishes with acetone, as acetone will destroy plastic. Allow the fixing agents to evaporate from your slides before continuing.

- Encircle your sections with a PAP-pen (see Fig. 1.4a).
- Rehydrate your slides for 30 min in TBS.
- Block for 30 min with 5 % normal donkey serum in TBS with 1 % BSA and 0.2 % Triton X-100 (see Fig. 1.4b).

  DO NOT RINSE; PROCEED TO PRIMARY ANTIBODY

- Incubate overnight at 4 °C with rabbit-anti-phospho-H2AX 1:500 in TBS with 1 % BSA.
- The following day, rinse your slides three times in TBS, and leave the last rinse for 30 min.
- Incubate for 1 h at room temperature with secondary antibody: Cy3-conjugated donkey-anti-rabbit 1:600 in TBS.
- Rinse your slides three times in TBS, and leave the last rinse for 30 min.
- Stain nuclei with Hoechst (1 mg/ml stock) 1:3000 in TBS for 5 min at room temperature.
- Mount slides with Fluoromount W.
- Let your slides dry at room temperature in the dark.
- You can check your slides under the microscope the next day.

1.2.2.2 Alternative Procedure to Analyse Multiple Markers on Cryosections

In this section we provide a protocol to stain against phospho-H2AX, whilst at the same time analysing hypoxia, through a staining against pimonidazole [7], vessels and all nuclei.