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Identification of Cells with Markers of Cellular Senescence in Human Formalin-fixed, Paraffin-embedded Brain Tissue Sections --Manuscript Draft--

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Abstract:	<p>Cellular senescence is a terminal arrest in proliferation associated with aging and age-related diseases. Senescent cells are characterized in vitro by several morphological and biochemical markers, such as flattened morphology, Senescence-Associated beta-galactosidase activity, chromatin rearrangements, and expression of the cyclin-dependent kinase-inhibitors p21 and p16INK4A.</p> <p>Here we describe a procedure to detect p16INK4A-positive cells by immunofluorescence on formalin-fixed, paraffin-embedded, human-brain specimens. Briefly, tissue slides are deparaffinated, rehydrated, and subjected to antigen-retrieval with standard immunohistochemistry procedures. The slides are then incubated with serum to block non-specific binding sites, and with primary antibodies against p16INK4A and a specific cell type marker. Finally, the slides are stained with fluorescently-conjugated secondary antibodies and counterstained with DAPI. This technique has been used successfully to detect p16INK4A positive astrocytes in human brains, but can be easily applied to other cell types, markers, and tissues, provided no cross-reactivity ensues between the antibodies.</p> <p>Our technique allows for the identification of subpopulations of senescent cells more rigorously than immunohistochemistry and enables the user to measure the abundance of a senescence marker semi-quantitatively.</p>
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Author Comments:	Thanks for the invitation



Drexel University College of Medicine

In the tradition of Women's Medical College of Pennsylvania and Hahnemann Medical College

Department of Pathology and Laboratory Medicine

Allison Diamond

Associate Editor, Journal of Visualized Experiments (JoVE)

Dear Ms. Diamond,

Enclosed is a manuscript by Crowe, Bitto, Bhat, Johnson, Trojanowski, Sell and myself entitled: "Identification of cells with markers of cellular senescence in human formalin-fixed, paraffin-embedded brain tissue sections".

Recently we demonstrated for the first time the presence of senescent astrocytes in human brain, during aging and as component of the brain of patients suffering from Alzheimer's disease (Bhat et al. 2012, PLoS One. 2012;7(9):e45069). We have proposed that the presence of senescent astrocytes contributes to the pathogenesis of AD and may represent a link between the aging process and progression of the disease. In previous studies, we identified p16^{INK4a} as a major protein up-regulated during astrocyte senescence (Bitto et al., Exp Cell Res. 2010:2961-8), and we have used this marker to identify senescent astrocytes in the archived postmortem human brain. In the manuscript, we describe the protocol for the detection of p16^{INK4a}-positive astrocytes by double immunofluorescence in human formalin-fixed, paraffin-embedded brain sections. The protocol involves several stages including tissue preparation, immunological detection, imaging and quantitation; and we feel that the opportunity to methodologically illustrate and accurately evaluate senescent cells in human brain tissue fits with JoVE's aims and unique multimedia format.

Senescent cells have been shown to accumulate in human tissues during aging and to underlie aging-related disease phenotypes; however, little information is available regarding the detection of senescent cells in human brain tissue. Therefore, we believe that a method to identify and evaluate the proportion of senescent astrocytes in human brain tissues could serve as the basis for investigating the consequences of their presence in a diverse set of pathological brain conditions.

Author contributions: protocol design and standardization EPC, AB, RB, CT. Data analysis: EPC, AB, FBJ, JQT, CS, CT. Manuscript writing: EPC, AB, CT.

I hope you will find our manuscript suitable for publication in JoVE.

Best regards,

Claudio Torres, Ph.D.

Assistant Professor

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Identification of Cells with Markers of Cellular Senescence in Human Formalin-fixed, Paraffin-embedded Brain Tissue Sections

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Keywords

Immunofluorescence, p16^{INK4A}, brain, senescence, neuroscience, astrocytes, aging

Short Abstract

We describe an immunofluorescence procedure to detect markers of cellular senescence in human formalin-fixed, paraffin-embedded brain sections. In contrast to immunohistochemistry, immunofluorescence permits semi-quantitative measurements and multiple staining on the same slide, which allows detecting different cell types more rigorously and assessing the abundance of senescence markers more objectively.

Long Abstract

Cellular senescence is a terminal arrest in proliferation associated with aging and age-related diseases. Senescent cells are characterized in vitro by several morphological and biochemical markers, such as flattened morphology, Senescence-Associated beta-galactosidase activity, chromatin rearrangements, and expression of the cyclin-dependent kinase-inhibitors p21 and p16^{INK4A}.

Here we describe a procedure to detect p16^{INK4A}-positive cells by immunofluorescence on formalin-fixed, paraffin-embedded, human-brain specimens. Briefly, tissue slides are deparaffinated, rehydrated, and subjected to antigen-retrieval with standard immunohistochemistry procedures. The slides are then incubated with serum to block non-specific binding sites, and with primary antibodies against p16^{INK4A} and a specific cell type marker. Finally, the slides are stained with fluorescently-conjugated secondary antibodies and counterstained with DAPI. This technique has been used successfully to detect p16^{INK4A} positive astrocytes in human brains, but can be easily applied to other

cell types, markers, and tissues, provided no cross-reactivity ensues between the antibodies.

Our technique allows for the identification of subpopulations of senescent cells more rigorously than immunohistochemistry and enables the user to measure the abundance of a senescence marker semi-quantitatively.

Introduction

Cellular senescence is a terminal arrest in cell proliferation¹ in response to telomere attrition, DNA damage, oncogenic activation, and oxidative stress^{2,3}. Cells expressing markers of senescence have been found *in vivo* in skeletal muscle, lung and liver of old animals⁴ and recently we have demonstrated the presence of senescent astrocytes in human brain⁵. Senescent cells contribute to the aging process and to the onset of age-related pathologies by secreting several pro-inflammatory cytokines⁶, a phenomenon known as Senescence-Associated Secretory Phenotype (SASP)⁷. At the cellular level, senescence is characterized by several distinctive markers, such as senescence-associated β -galactosidase activity⁸, accumulation of heterochromatin protein 1 and of the histone variant macro H2A^{9,10}, secretion of interleukin 6 and matrix metalloproteinase 1^{5,7}, and increased expression of the cyclin-dependent kinase-inhibitor p16^{INK4A}. Although these markers are not exclusive to senescent cells, expression of p16^{INK4A} is considered to be a robust indicator of cellular senescence *in vivo*¹¹ and has been used successfully in human specimens^{5,11}. p16^{INK4A}-expressing cells appear to be a critical factor in age-related pathologies: p16^{INK4A}-positive astrocytes accumulate in human brains affected by Alzheimer's disease⁵, and clearing p16^{INK4A}-expressing cells delays aging in rodents¹².

We describe here a method for detecting cells expressing p16^{INK4A} in formalin-fixed, paraffin-embedded (FFPE), human-brain specimens by immunofluorescence⁵. Our protocol offers several advantages in comparison to immunofluorescence on frozen sections or immunohistochemistry: *i*) it can be used on tissues from most pathology autopsy archives (we provide an example on frontal cortex autopsy-specimen archival-tissue), *ii*) it is suitable for multiple staining on the same tissue slide, thus allowing discrimination between cell types based on the expression of specific cell markers, *iii*) it can be coupled with regular immunohistochemistry and thus with staining protocols clinically approved for diagnostic use.

Protocol

Human Subjects Ethics Statement

This research was performed in compliance with and following approval by the Institutional Review Board at Drexel University College of Medicine (Protocol Number: 18172), and the Institutional Review Board at University of Pennsylvania (Protocol Number: 180600). All tissue samples were de-identified and no protected health information was made available to the researchers; therefore, this protocol was approved as exempt study and consent was waived.

1) Deparaffination and Rehydration of Tissue Sections

Note: Once the protocol has been started, ensure that the slides are never allowed to dry out because this could interfere with quality of the staining.

1.1) Deparaffinize tissue sections on glass slides by immersing slides into a glass dish filled with xylene for 5 minutes (CAUTION: Use only under a chemical fume hood, possible cancer hazard). Transfer slides to the next dish filled with fresh xylene and incubate for 5 minutes and repeat this step once more for a total of 3 incubations of 5 minutes each. Keep track of how often the xylene in each container was used and properly discard of xylene after 3 uses.

Note: Set up and turn on heat steamer as described in point 2.2

1.2) Transfer slides to the next dish filled with 100% alcohol (CAUTION: flammable) and wash for 5 minutes. Repeat once more with fresh 100% alcohol for a total of 2 X 5-minute washes.

1.3) Prepare 90% alcohol by diluting with deionized water (i.e. 90 ml of alcohol + 10 ml of deionized water). Incubate slides in 90% Reagent Alcohol for 5 minutes. Repeat once more with fresh 90% alcohol for a total of 2 X 5-minute washes.

1.4) Prepare 80% alcohol by diluting with deionized water. Incubate slides in 80% alcohol for 5 minutes. Repeat once more with fresh 80% alcohol for a total of 2 X 5-minute washes.

1.5) Prepare 70% alcohol by diluting with deionized water. Incubate slides in 70% Reagent Alcohol for 5 minutes. Repeat once more with fresh 70% alcohol for a total of 2 X 5-minute washes.

1.6) Incubate slides in deionized water for 5 minutes. Repeat once more with fresh deionized water for a total of 2 X 5-minute washes.

2) Antigen Retrieval

2.1) Transfer slides to a horizontal slide holder filled with 10 mM citrate buffer pH 6.0. Equilibrate slides in this buffer for 5 minutes. Prepare 10 mM citrate buffer pH 6.0 by diluting from 100 mM stock of citrate buffer. For 1L of 100 mM citrate buffer stock pH 6.0, dilute 21.01g of citric acid monohydrate (formula weight 210.14 g/mol) in distilled deionized water, bring the pH to 6.0 with a solution of NaOH, and then adjust the volume to 1L.

2.2) Heat-steam slides in a covered dish filled with citrate buffer for 15-20 minutes depending on the degree of cross-linking of the tissue sample. Heat steamer (rice cooker) should be set-up and turned on during deparaffination steps by filling lower chamber approximately halfway with distilled water.

2.3) Remove covered dish with slides in citrate buffer from heat (CAUTION: dish will be hot) and place on lab bench top for at least 30 minutes.

2.4) Wash slides 2 X 5 minutes in 1X PBS 0.1% BSA solution. (For 1 L of 1X PBS-0.1%BSA solution, add 1 g of bovine serum albumin (BSA) to 1 L of phosphate-buffered saline (1X PBS) and mix well to dissolve BSA).

3) Blocking

3.1) Prepare a humidified chamber by placing filter paper in the bottom of a plastic slide box and moistening with deionized water.

3.2) Prepare blocking solution to a final concentration of 0.1% BSA (w/v) 0.25% Triton X-100 (v/v), 5% normal goat serum (v/v), and 5% normal donkey serum (v/v) in 1X PBS. The type of serum used in the blocking solution will depend on the species in which the secondary antibodies were raised (i.e. If using donkey anti mouse secondary antibody and goat anti rabbit secondary, block with donkey and goat serums simultaneously).

3.3) Remove slides one at a time from the PBS-BSA wash and wipe away excess liquid from the back and edges of the slide. Avoid making any contact with the tissue section.

3.4) Place slide in humidified chamber. Draw a hydrophobic border around the outside of the tissue section with a PAP pen. When circumscribing tissue area, ensure that area through which the line is drawn is completely dry; otherwise the border may be lost during immunostaining. Avoid putting too much pressure on the pen, which could result in excess solution being discharged onto the slide.

3.5) Immediately add enough blocking solution to cover the tissue. Use approximately 100-200 microliters per tissue section, depending on the surface area. Avoid contacting the tissue section with the pipette tip. Repeat for all slides.

3.6) Incubate slides with blocking solution for 90 minutes in the humidified chamber at room temperature.

4) Immunofluorescence Staining with Two Primary Antibodies Simultaneously

4.1) For immunostaining with more than one primary antibody simultaneously, (i.e. a senescence biomarker (mouse anti-p16^{INK4A}) and a cellular biomarker (rabbit anti-GFAP (glial fibrillary acidic protein) to visualize astrocytes), dilute primary antibodies to desired final concentration in antibody dilution buffer consisting of 1X PBS, 0.1% BSA (w/v), and 0.25% Triton X-100 (v/v) in the same 1.5 ml tube and mix well. See Discussion regarding selection of primary antibodies.

4.2) Blot off blocking solution by pressing the edge of the slide against filter paper.

4.3) Apply the solution of primary antibodies to each slide. Use approximately 75 microliters per slide or enough volume to cover depending on tissue surface area.

4.4) Incubate in humidified chamber overnight (approximately 16 hours) at 4 degrees Celsius. Handle humidified chamber carefully to avoid displacing primary antibody solution from the tissue area.

4.5) Blot off primary antibody by touching the edge of the slide to filter paper. Wash slides in 1X PBS 0.1% BSA for total of 3 x 5-minute washes.

4.6) Prepare secondary antibodies (conjugated to different fluorochromes) by diluting to a final concentration of 1: 500 in antibody dilution buffer (see step 4.1). Incubate in the dark in a humidified chamber for 1 hour. The secondary antibodies are light-sensitive; therefore, this step and all subsequent steps should be protected from light.

4.7) Blot off secondary antibody solution by touching the edge of the slide to filter paper. Wash slides in 1X PBS 0.1% BSA for total of 3 x 5-minute washes.

4.8) Prepare DAPI working solution by diluting DAPI (50ug/ml stock) 1:5000 with 1X PBS 0.1% BSA. Apply the DAPI working solution to the slides and incubate for 10 minutes in the dark.

4.9) Rinse slides abundantly with at least 3-4 washes with distilled deionized water.

5) Mounting Slides for Immunofluorescence Microscopy

5.1) Remove slides one at a time from the water wash and wipe away excess liquid from the back of the slide. Avoid making any contact with the tissue section.

5.2) Place a drop (approximately 10 microliters) of fluorescence mounting medium in the center of the tissue section. Avoid using too much mounting medium, otherwise the coverslip will move against the tissue section and it will be difficult to seal the edges.

5.3) Place a glass coverslip (24 x 50 mm) on edge of slide and slowly lower onto the tissue section to avoid generating bubbles.

5.4) Seal coverslips with clear nail polish by first putting a drop of nail polish at one corner of the slide with coverslip and dry undisturbed for approximately 5 minutes. Seal the remaining corners with nail polish and then seal the short and long edges of the coverslip. Allow nail polish to dry before going to the microscope.

5.5) Store stained slides at 4 degrees Celsius and visualize within two weeks.

6) Image Acquisition and Analysis

Using a fluorescence microscope, capture images using the appropriate channels depending on the fluorochromes used.

OPTIONAL: Combined Immunohistochemistry and Immunofluorescence

Prior to immunofluorescence staining, immunohistochemistry can be performed in the same tissue section for an additional marker. Following antigen retrieval (Step 2), sections can be stained using standard immunohistochemical methods in the absence of a counterstain. Slides are visualized with the fluorescence microscope and captured in brightfield.

Representative Results

We demonstrate a method for detecting a senescence biomarker within a specific cell type in situ in archived FFPE human brain tissue. Figure 1 shows representative images of p16^{INK4A} and GFAP staining in AD patient and an age-matched control subject. We demonstrate that p16^{INK4A} staining is diffusely localized throughout the nucleus, while GFAP staining is cytoplasmic. Nuclear p16^{INK4A} staining is considered positive when it colocalizes with DAPI and the signal intensity is markedly elevated compared with the background levels. Nuclei are scored as either positive or negative for p16^{INK4A} and the number of astrocytes that are positive for p16^{INK4A} is expressed as percent of the total number of GFAP-positive cells. High background levels of staining (Figure 2) may preclude proper analysis and identification of senescent cells in tissue sections.

Figure Legends:

Figure 1: p16^{INK4A} and GFAP immunofluorescence staining in FFPE sections of human brain tissue. Formalin-fixed paraffin-embedded sections from the frontal cortex

of an Alzheimer's disease patient (top panels) or an age-matched control subject (bottom panels) were probed for DAPI (blue) to visualize the nuclei, p16^{INK4A} (red) to identify senescent cells, and GFAP (green) to identify astrocytes. Arrow denotes p16^{INK4A}-positive (senescent) astrocyte.

Figure 2: Suboptimal results of p16^{INK4A} and GFAP staining. Representative images of a section with high background level of p16^{INK4A} staining (red).

Discussion

Double immunofluorescence labeling of human brain tissues is a well-established method in the study of neurodegenerative disease¹³⁻¹⁵ and methods for performing immunofluorescence on FFPE tissues have been described previously¹⁶, thus we redirect the reader to them for a more detailed discussion of the technique, while we will focus on key concepts necessary for applying the technique successfully to detect senescent cells. Background signal and antibody specificity are the most critical challenges to using our method successfully. Background fluorescence in FFPE slides is inherently higher than in frozen sections or individual cells¹⁶, thus it is of paramount importance to distinguish between antibody signal and auto-fluorescent structures in the specimen in use. In order to overcome this issue, we strongly encourage the user to acquire images at wavelengths where the fluorochromes in use have little to no emission intensity, in addition to those dedicated to the specific signals: structures that emit a signal in all channels are likely to be auto-fluorescent, while those emitting only in dedicated channels are specific to the antibodies used. Additionally, images acquired from unstained slides should also reveal what structures are likely to show background signal.

FFPE sections can present several non-specific epitopes to both primary and secondary antibodies because of their complex architecture and their high degree of cross-linking. Therefore, the specificity of each antibody and antibody combination should be tested on each new tissue used. In particular, we strongly encourage the user to run a pilot staining with each primary antibody individually and to stain a slide with only the secondary antibodies. If the specificity of an antibody remains uncertain, we suggest preparing a negative control slide, where the antibody in question has been incubated with an excess concentration of blocking peptide, when the latter is available, or testing the antibody on tissues that should not express the antigen. Furthermore, non-specific signal could come from particles and impurities accumulating on the slides during the staining protocol; in order to minimize this phenomenon, all solutions should be prepared fresh and filtered before use.

This protocol is suitable for the detection of cells demonstrating biomarkers of senescence within FFPE tissue sections, particularly those composed of several cell

types. We have optimized our protocol to detect a senescence biomarker (p16^{INK4A}) within the nucleus of a cell that is positive for a cellular marker (GFAP). The protocol can be expanded to examine additional biomarkers of senescence as well as other cell types when antibodies are available, provided a few requirements are met. First, the primary antibodies to be used in combination must be raised in different species, in order to avoid cross-reactivity of the secondary antibodies. Although sequential staining techniques have been successfully used in frozen tissues and fixed cells, we have had limited success in implementing them on FFPE sections. Different IgG isotypes have been successfully used in combination for staining FFPE elsewhere¹⁶ and could thus overcome this limitation. Secondly, secondary antibodies should be carefully selected to avoid cross-reactivity with the other secondary antibody; for example, secondary antibodies raised in goat should not be used in combination with a primary antibody raised in the same species, in order to avoid cross-reactivity with the anti-goat secondary antibody. A vast array of fluorescently-tagged antibodies is available from several commercial providers, thus we strongly encourage the user to invest in additional antibodies instead of relying on sequential staining and extra blocking steps to overcome this issue.

The quality of the archived brain tissue specimen is also a key factor in the outcome of the staining. Factors such as post-mortem interval and degree and type of fixation should be taken into consideration when selecting tissues for study. In studies with archived brain tissue from neurodegenerative disease subjects, it may be informative to compare a region known to be affected with a region that is relatively unaffected in the course of disease; for example, comparing frontal cortex with cerebellum in Alzheimer's disease patients.

While this manuscript was in review, a publication by Sawicka et al. described discrepancies in staining between the anti-p16^{INK4A} antibody (clone F-12) described in our protocol and three other commercially available antibodies: in particular, only the F-12 antibody produced a nuclear staining pattern and was unresponsive to RNA interference against p16^{INK4A}¹⁷. Such differences may be due to the existence of several different gene products of the CDKN2A locus: in fact, nuclear localization of p16^{INK4A} has been described independently of the F-12 antibody¹⁸, and no F-12 immuno-reactivity was detected in tumor samples bearing a deletion in the chromosome arm carrying the CDKN2A locus¹⁹. Furthermore, we find that F-12 immuno-reactivity correlates with age of the donor, age-related pathological conditions, and other senescence-associated markers (⁵ and unpublished observations), thus making the antibody suitable for detecting senescent cells.

Acknowledgements

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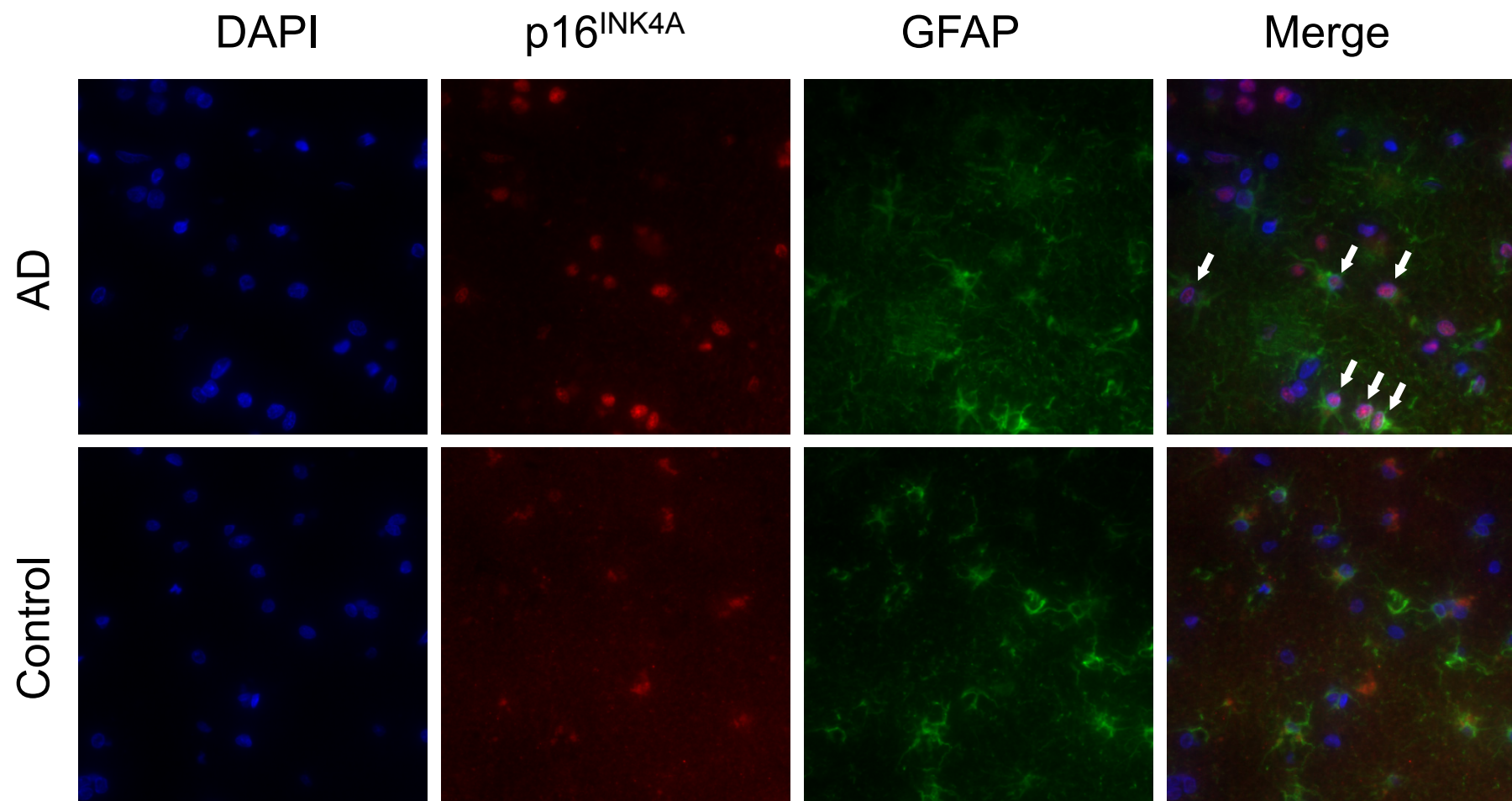
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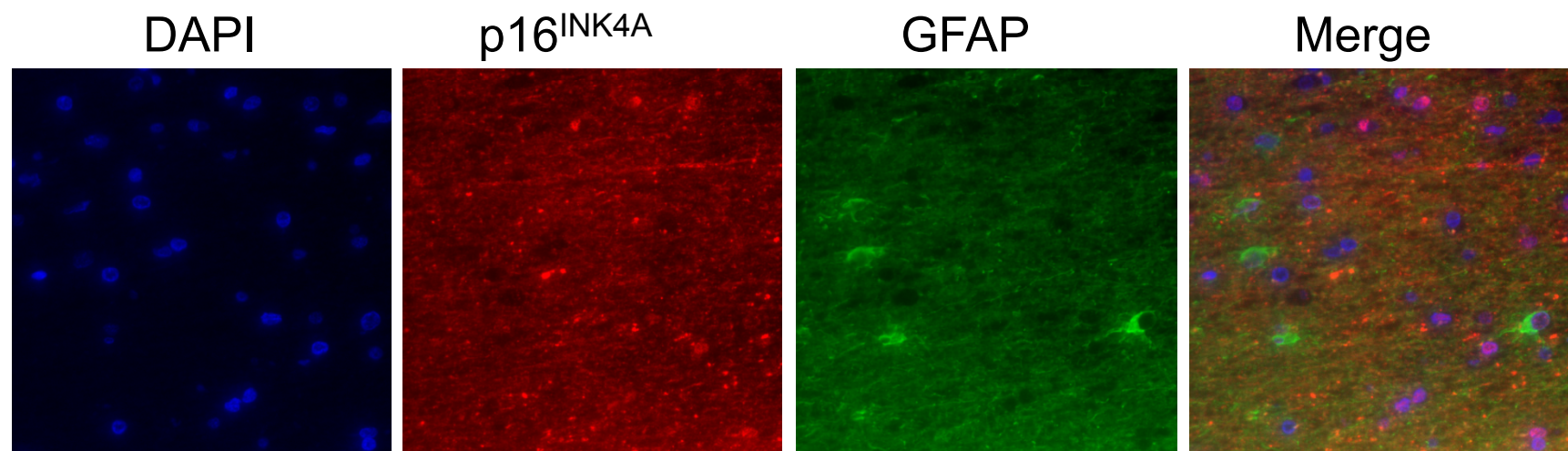
The authors declare that they have no competing financial interests.

References

- 1 Hayflick, L. The limited *in vitro* lifetime of human diploid strains. *Exp. Cell Res.* **37**, 614-636 (1965).
- 2 Rodier, F. & Campisi, J. Four faces of cellular senescence. *The Journal of cell biology* **192**, 547-603, doi:10.1083/jcb.201009094 (2011).
- 3 Iwasa, H., Han, J. & Ishikawa, F. Mitogen-activated protein kinase p38 defines the common senescence-signalling pathway. *Genes Cells* **8**, 131-144, doi:620 [pii] (2003).
- 4 Kreiling, J. *et al.* Age-associated increase in heterochromatic marks in murine and primate tissues. *Aging Cell* **10**, 292-304, doi:10.1111/j.1474-9726.2010.00666.x (2011).
- 5 Bhat, R. *et al.* Astrocyte senescence as a component of Alzheimer's disease. *PLoS One* **7**, doi:10.1371/journal.pone.0045069 (2012).
- 6 Campisi, J., Andersen, J. K., Kapahi, P. & Melov, S. Cellular senescence: a link between cancer and age-related degenerative disease? *Seminars in cancer biology* **21**, 354-359, doi:10.1016/j.semcancer.2011.09.001 (2011).
- 7 Coppe, J. P. *et al.* A role for fibroblasts in mediating the effects of tobacco-induced epithelial cell growth and invasion. *Molecular cancer research : MCR* **6**, 1085-1098, doi:6/7/1085 [pii] 10.1158/1541-7786.MCR-08-0062 (2008).
- 8 Dimri, G. P. *et al.* A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 9363-9367 (1995).
- 9 Zhang, R. *et al.* Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. *Developmental cell* **8**, 19-30, doi:10.1016/j.devcel.2004.10.019 (2005).
- 10 Adams, P. D. Remodeling of chromatin structure in senescent cells and its potential impact on tumor suppression and aging. *Gene* **397**, 84-93, doi:S0378-1119(07)00190-4 [pii] 10.1016/j.gene.2007.04.020 (2007).
- 11 Ressler, S. *et al.* p16INK4A is a robust in vivo biomarker of cellular aging in human skin. *Aging Cell* **5**, 379-389, doi:10.1111/j.1474-9726.2006.00231.x (2006).
- 12 Baker, D. *et al.* Clearance of p16(Ink4a)-positive senescent cells delays ageing-associated disorders. *Nature* (2011).
- 13 Neumann, M. *et al.* Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* **314**, 130-133, doi:10.1126/science.1134108 (2006).

- 14 Uryu, K. *et al.* Concomitant TAR-DNA-binding protein 43 pathology is present in Alzheimer disease and corticobasal degeneration but not in other tauopathies. *Journal of neuropathology and experimental neurology* **67**, 555-564, doi:10.1097/NEN.0b013e31817713b5 (2008).
- 15 Irwin, D. J. *et al.* Acetylated tau, a novel pathological signature in Alzheimer's disease and other tauopathies. *Brain : a journal of neurology* **135**, 807-818, doi:10.1093/brain/aws013 (2012).
- 16 Robertson, D., Savage, K., Reis-Filho, J. S. & Isacke, C. M. Multiple immunofluorescence labelling of formalin-fixed paraffin-embedded (FFPE) tissue. *BMC cell biology* **9**, 13, doi:10.1186/1471-2121-9-13 (2008).
- 17 Sawicka, M. *et al.* The Specificity and Patterns of Staining in Human Cells and Tissues of p16INK4a Antibodies Demonstrate Variant Antigen Binding. *PLoS One* **8**, doi:10.1371/journal.pone.0053313 (2013).
- 18 Lukas, J. *et al.* Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. *Nature* **375**, 503-506, doi:10.1038/375503a0 (1995).
- 19 Haller, F. *et al.* Loss of 9p leads to p16INK4A down-regulation and enables RB/E2F1-dependent cell cycle promotion in gastrointestinal stromal tumours (GISTs). *The Journal of pathology* **215**, 253-262, doi:10.1002/path.2352 (2008).





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Xylene	Fisher Scientific
Reagent Alcohol	Fisher Scientific
Citric acid monohydrate	Sigma Aldrich
Phosphate-buffered Saline	Mediatech
Bovine Serum Albumin	Gemini Bioproducts
Triton X-100	Sigma Aldrich
ImmEdge Pen (PAP pen)	Vector Laboratories
Goat Serum	MP Biomedicals
Donkey Serum	Millipore
Mouse anti p16 antibody	Santa Cruz Biotechnology
Rabbit anti GFAP antibody	Millipore
Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L)	Life Technologies
Alexa Fluor 555 Donkey Anti-Mouse IgG (H+L)	Life Technologies
DAPI (4',6-Diamidino-2-phenylindole dihydrochloride)	Sigma Aldrich
Vectashield Mounting Medium	Vector Laboratories
Coverslips	Fisher Scientific
Clear nail polish	Any supplier
Heat steamer	Any supplier
Olympus BX61 fluorescence microscope	Olympus
Hamamatsu ORCA-ER camera	
Slide Book 4 Software	Intelligent Innovations, Inc

Catalog Number	Comments
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A962F-1GAL	flammable liquid
C7129-500G	eye irritant
46-013-CM	10X
700-101P	
T-9284	irritant
H-4000	
191356	
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Article Title:

Identification of Cells with Markers of Cellular Senescence in Human FFPE Brain Tissue Sections

Signature:

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Department of Pathology and Laboratory Medicine

Allison Diamond

Associate Editor, Journal of Visualized Experiments (JoVE)

Manuscript JoVE50520R1

Dear Ms. Diamond,

Enclosed is the revisited version of our manuscript entitled "Identification of Cells with Markers of Cellular Senescence in Human Formalin-fixed, Paraffin-embedded Brain Tissue Sections."

We thank the reviewers for their careful review and thoughtful comments on our manuscript. We have carefully taken their comments into consideration in preparing our revision, which has resulted in a paper that is clearer and meets JoVE editorial standards. In addition, we have included an additional paragraph at the end of the discussion section in the manuscript detailing our rationale for use of the F-12 clone of the p16^{INK4a} antibody as a senescence biomarker. The following summarizes how we responded to reviewer/editorial comments in a point-by-point manner.

Editorial comments:

1) Comment: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Please remove all commercial sounding language from your manuscript. All commercial products should be sufficiently referenced in the table of materials/reagents. Specifically, please remove any TM/R/C symbols in your table of materials/reagents.

1) Response: We have removed all instances of commercial-sounding language from the manuscript. Instances of the removal of brand names from the manuscript are highlighted using track changes. TM/R/C symbols were removed from the table of materials/reagents.

Reviewers' comments:

Reviewer #1:

1) There were no additional points from Reviewer #1 to be addressed.

Reviewer #2:

1) Comment: It would be nice to see the comparison with frozen sections of the same patient, but I can imagine that it is not so straightforward to have them.

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Department of Pathology and Laboratory Medicine

1) Response: The authors would like to thank Reviewer #2 for the suggested comparison of fixed and frozen tissues from the same patient. Unfortunately, we have been unable to obtain both types of tissue from the same patient in our current studies.

Reviewer #3:

1) There were no additional points from Reviewer #3 to be addressed.

I hope you will find our manuscript suitable for publication in JoVE.

Best regards,

Claudio Torres, Ph.D.
Assistant Professor

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