Progress in Retinal and Eye Research 48 (2015) 181–202

Contents lists available at ScienceDirect

Progress in Retinal and Eye Research

journal homepage: www.elsevier.com/locate/prer

Fixation strategies for retinal immunohistochemistry

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A R T I C L E I N F O

Article history:
Received 9 February 2015
Received in revised form
6 April 2015
Accepted 6 April 2015
Available online 17 April 2015

Keywords:
Formaldehyde
Glutaraldehyde
Retina
Fixatives
Antigen retrieval
Quenching
Sucrose
pH

A B S T R A C T

Immunohistochemical and *ex vivo* anatomical studies have provided many glimpses of the variety, distribution, and signaling components of vertebrate retinal neurons. The beauty of numerous images published to date, and the qualitative and quantitative information they provide, indicate that these approaches are fundamentally useful. However, obtaining these images entailed tissue handling and exposure to chemical solutions that differ from normal extracellular fluid in composition, temperature, and osmolarity. Because the differences are large enough to alter intercellular and intracellular signaling in neurons, and because retinae are susceptible to crush, shear, and fray, it is natural to wonder if immunohistochemical and anatomical methods disturb or damage the cells they are designed to examine. Tissue fixation is typically incorporated to guard against this damage and is therefore critically important to the quality and significance of the harvested data. Here, we describe mechanisms of fixation; advantages and disadvantages of using formaldehyde and glutaraldehyde as fixatives during immunohistochemistry; and modifications of widely used protocols that have recently been found to improve cell shape preservation and immunostaining patterns, especially in proximal retinal neurons.
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Abbreviations: cAMP, 3′-5′-cyclic adenosine monophosphate; GPI, glycosylphosphatidylinositol; HCN, hyperpolarization-activated cyclic nucleotide-gated; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SNR, signal-to-noise ratio.

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[1] Percentage of work contributed by each author in the production of the manuscript is as follows: Tyler W. Stradleigh: 60%; Andrew T. Ishida: 40%.

http://dx.doi.org/10.1016/j.preteyeres.2015.04.001
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1. Introduction

Structural studies that began more than a century ago have shown that retinal cell types differ in shape, dimensions, distribution, connections, and protein expression (Marc, 2008; Marc et al., 2013; Masland, 2001; Polyak, 1941; Ramón y Cajal, 1893; Siegert et al., 2009; Stell, 1972; Walls, 1963; Wässle, 2004). These studies, like those of other tissues, have relied on procedures termed "fixation" to preserve phenotypic properties in morphologically and chemically life-like states (Hopwood, 1985). Ideally, fixation stops physiological responses and metabolic processes within cells, and reduces tissue damage and distortion due to mechanical manipulation and osmotic stress during subsequent processing. Fixation is particularly helpful when examining fragile specimens, and it is necessary when the analytical methods are incompatible with live cells.

Successful fixation preserves tissue rapidly to capture its structure and state at the moment of exposure to the fixing medium, and for periods that permit *post-hoc* analysis. Although fixation should preserve both the natural shape of cells and the *in vivo* organization of tissue components (e.g., proteins), existing fixation protocols often strike compromises between morphological preservation and maintenance of the normal chemical environment (Eltoum et al., 2001). Moreover, although well-fixed preparations can be recognized on the basis of gross tissue dimensions and cell morphologies, it is less clear whether a given protocol accurately captures other properties of the same cells, whether the same protocol preserves the phenotypes of all cells, and whether a single protocol can clearly identify cells despite differences in species, age, and/or health.

Sections 2 and 3 describe mechanisms of fixation and immunohistochemistry. Sections 4 and 5 then focus on protocols that use chemical fixatives to define the morphology, organization, and light responses of retinal neurons at the light microscopic level, discussing both positive and negative impacts of chemical fixation on results obtained by immunohistochemistry. Lastly, Section 6 describes alternative fixation strategies that have recently been found to improve the pattern and intensity of immunostaining in proximal neurons of adult mammalian retinae. Three conclusions are supported. Firstly, formaldehyde has enabled investigators to detect and localize a larger variety of molecules that contribute to light sensitivity, synapses, and signal generation in the retina than any other fixative. Secondly, formaldehyde alone is not the best chemical fixative in multiple respects for certain studies. Thirdly, protocols that use formaldehyde-based fixatives can be modified in various ways to improve the preservation of cell phenotypes. Although we are unaware of previous reviews of these topics, we refer readers to two particularly helpful websites (Fisher, 2013; Marc, 2014) and to comparisons of eyes and retinae after fixation by various protocols (Chidlow et al., 2011; Eldred et al., 1983; Hageman and Johnson, 1991; Izumi et al., 2000; Latendresse et al., 2002; Margo and Lee, 1995; McMenamin, 2000; Peichl, 1989; Rasmussen, 1974; Webster et al., 1969) for additional information.

2. Mechanisms of fixation

Chemical and physical methods of fixation have been developed over more than a century of histological work. The most widely used fixatives are chemical, and these are classified as either crosslinking or coagulant based on their mode of action and effects on soluble proteins.

Crosslinking, non-coagulating chemical fixatives confer structural support without directly changing the overall solubility of individual proteins, linking adjacent tissue structures instead through intermediate molecules. Crosslinks occur at specific target regions, depending on the crosslinking agent used, and the crosslinked macromolecular complexes may have altered water solubility. Crosslinking fixatives include aldehydes and carbodiimides, with aldehydes preferentially crosslinking free amino groups on amino acid chains, and carbodiimides tending to crosslink adjacent carbonyl groups (Hopwood, 1985). Aldehydes are the most common crosslinking fixatives, with formaldehyde generally used for light microscopy and glutaraldehyde generally used for electron microscopy. They differ in their reaction speeds, rates of tissue penetration, avidity for non-protein molecules and, as discussed below, practical advantages and disadvantages. Carbodiimide has rarely been used in published retinal studies (Gastinger et al., 1999; Haverkamp and Wässle, 2000; Ivanova et al., 2013) and will not be considered further here.

Coagulant fixatives decrease protein solubility and initiate protein precipitation from solution, fixing precipitated proteins in place (Boon and Kok, 2008). Coagulant fixatives are fast-acting, so much so that when combined with formaldehyde, the coagulant components are thought to serve as the primary fixative while formaldehyde stabilizes the precipitated proteins into place (Wenk, 2006). Coagulant fixatives comprise two general types: dehydrating fixatives and acidic fixatives (Wenk, 2006).

Dehydrating coagulant fixatives remove the layer of water that normally separates adjacent amino acid chains in live tissue. This initiates intramolecular and intermolecular interactions that encourage spontaneous changes in protein structure and, in turn, water solubility (Boon and Kok, 2008). Ethanol, methanol, and acetone are commonly used dehydrating coagulants. Ethanol has been found to preserve structural features of human eyes at a gross level (Karma et al., 2007; Krauss, 1990). Acetone has been used as a

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