Glutaraldehyde: A review of its fixative effects on nucleic acids, proteins, lipids, and carbohydrates

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Abstract

Tissue banking methods such as brain banking often face a trade-off between morphological and molecular preservation. For example, cryopreservation is preferred for subsequent molecular assays, while fixation by aldehyde crosslinking is commonly used for microscopy. Among aldehyde fixatives, formaldehyde is often considered better for immunohistochemistry, while glutaraldehyde is often considered better for electron microscopy. However, it is unclear whether morphological versus molecular preservation trade-offs reflect fundamental biology or technology limitations. As a window into this discussion, in this narrative review, I evaluate the literature regarding the effects of glutaraldehyde on molecular preservation, with an emphasis on nervous system tissue. Available evidence suggests that crosslinking with glutaraldehyde has minimal direct effects on most molecular features, with a few critical exceptions such as protein conformation. On the other hand, glutaraldehyde fixation frequently fails to retain non-directly crosslinked molecules such as lipid and carbohydrate species during subsequent dehydration steps. Further, as a result of probe diffusion limitations or strong covalent interactions with glutaraldehyde, many molecules can be more difficult to visualize or otherwise measure in tissue fixed with glutaraldehyde. As a practical guide for investigators that are considering using glutaraldehyde, I also point out representative molecular assays that have been performed in tissue fixed with glutaraldehyde, and how this set of assays could be improved and expanded in the future.

Introduction

In many mammalian tissue systems including the nervous system, both the intricate geometry of cells as well as their molecular constituents are essential in determining their function. For example, the kinetics of action potentials in neurons is affected by their three-dimensional cell membrane configurations as well as their expression of ionophoric proteins and countless other molecules. Morphological approaches are often not fully dispositive about the function of a structure. For example, lysosomes often require enzymatic or

immunologic identification in order to decisively identify them in an electron microscopy image (Maunsbach & Afzelius, 1999). As another example, the strength of a synapse likely cannot be perfectly predicted from its size, and instead depends also on other molecular properties, such as the number of ion channels, neurotransmitter receptors, and the contents of any observed vesicles (Kasai et al., 2003; Benito & Barco, 2010). Ideally, structure-function studies are best facilitated by preservation procedures that allow for the investigation of both morphologic and molecular features in the same sample. As a result, systems biology requires tools that allow for the study of both the morphological structure of cells as well as the molecules that they express and their biochemical properties, and there is an intense focus on the development of such tools. Often, the most important steps in the tissue preservation process are the initial steps, which affects how close the resulting structure is to its lifelike form, what the effects of subsequent processing steps or storage will be, and whether molecules will be lost, moved, chemically altered, or inaccessible.

One key trade-off that investigators often confront is choosing between preservation methods that are relatively better at facilitating the study of morphology, such as those that employ fixation via crosslinking aldehydes, and methods that are relatively better at preserving molecular features, such as those that employ cryopreservation (Tang et al., 2012; Shabihkhani et al., 2014). This trade-off may come about as a result of *intrinsic properties* of biological tissues, such as the lipids and proteins that make up cell membranes, that may need to be altered in some way to allow their morphology to retain a static shape that approximates their *in vivo* form. The trade-off may also come about as a result of *selection* by investigators for preservation procedures that facilitate the study of at least one of these critical parameters. Glutaraldehyde (GA) is an aliphatic, five-carbon, dialdehyde crosslinking agent (Hermanson, 2013) that falls on the morphological preservation side of this trade-off: it is typically considered to be particularly useful for morphologic studies such as electron microscopy and to be less useful for molecular studies such as enzyme assays and immunohistochemistry (Sabatini, Bensch & Barnett, 1963). However, it is unclear the degree to which these properties of GA are due to the intrinsic effects of GA on biological tissues, as opposed to limitations in the available protocols and tools for measuring molecules in cells and tissues that have been fixed by GA.

Because they will be used frequently throughout this review, it is important to offer definitions of morphology and molecular feature preservation. In this review, *morphology* is broadly defined to include all of the tissue-level and cellular-level features that are visible under the light and electron microscope. Notably, features visible under the electron microscope are generally referred to as “ultrastructure.” Tissues fixed with GA can be compared to *in vivo* ultrastructure by multiple methods, including freeze fracture electron microscopy that uses cryofixation of very thin tissue sections, as well as super-resolution light microscopy of live cells prior to fixation. These studies have generally found that

GA fixation induces few changes from the *in vivo* structural state, and is among the best morphologic preservation options available for larger tissue samples such as organs (Korogod, Petersen & Knott, 2015). However, it is important to note that aldehyde fixation in general, and GA fixation in particular, can result in several changes to *in vivo* morphology, some of which have been classified under the general umbrella of “fixation artifacts.” These include changes to membranes including vacuolization (Hobro & Smith, 2017); changes to organelles including flattening of synaptic vesicles (Gray, 1976); alterations of myelin sheath including myelin figures (Schultz & Case, 1970; Schultz & Wagner, 1986); and alterations of extracellular space (Korogod, Petersen & Knott, 2015). However, these morphologic artifacts caused by aldehyde fixation are outside the scope of this review.

In this review, a *molecular feature* will refer to any property of the trillions of molecules present in many mammalian tissues, including any property of the four major macromolecular subtypes: nucleic acids, proteins, lipids, and carbohydrates. There are three major types of molecular features that I will consider. The first is *spatial*, *i.e.* whether the molecule is in the same location as it was during the state prior to fixation. Spatial information preservation is on a spectrum; on one extreme, the molecule is in the exact same location within a cell and relative to other molecules; on the other extreme, it is not present in the tissue at all, *e.g.* as a result of having been washed out of the tissue during perfusion. The second component of molecular preservation is *chemical*, *i.e.* whether the chemical composition or conformation of a molecule has been altered during the fixation process. The third component of molecular preservation is *accessibility*, *i.e.* whether it is practical or possible to measure a molecule or a type of molecule after a fixation process. Accessibility could be altered in many ways as a result of fixation, for example as a result of lack of space for a molecular probe to diffuse into a cell, or as a result of an inability to dissociate molecules before profiling them. When an author claims that a fixation procedure will “*damage*” or “*destroy*” a particular molecule or component of a molecular such as an antigen, they could be referring to a deficit in any of these three components of molecular preservation. Where possible, this review will try to specify which type(s) of changes occur to particular molecular features as a result of the GA fixation process.

The morphological versus molecular preservation trade-off is of particular importance in human tissue banking, because of the scarcity of the donated tissue, the high degree of agonal and post-mortem damage that is frequently present, and the obvious value of human tissue for illuminating human disease processes. Despite the importance of tissue banking, the methods for preserving tissue samples, such as brains, is a relatively understudied research topic. One of the more unique challenges for human tissue banking is that at the time of preservation it is not possible to be sure of what studies will eventually be performed on this limited tissue source. As a result, tissue bankers must try to select preservation procedures that will broadly maximize the preservation of

cells and tissues in the brain for use in the future by investigators with diverse goals (Haroutunian & Davis, 2002). In brain banking, one approach frequently used to address the trade-off between morphologic and molecular preservation is to split the brain into two halves by making an incision at the midsagittal plane, and preserve one hemisphere with aldehyde fixation (Sheedy et al., 2008; Vonsattel et al., 2008; Nacul et al., 2014), and the other hemisphere via cryopreservation of dissected tissue blocks. While this is an effective and creative use of limited brain tissue, this solution presents its own challenges, including severing any inter-hemispheric connections, limiting the comparisons that can be made between hemispheres of the brain (King et al., 2015), and making perfusion fixation more technically challenging, albeit not impossible (Waldvogel et al., 2006; de Oliveira et al., 2012). Therefore, there is a need for improved brain banking procedures that do not entail as vexing a choice along the morphological-molecular axis.

The goal of this review is to evaluate the existing evidence regarding the effects of GA fixation on molecular features such as DNA, RNA, proteins, lipids, and carbohydrates. This review is similar to several other reviews and book chapters in the past (Hayat, 1981; Bullock, 1984; Griffiths, 1993; Kiernan, 2000, 2015; Migneault et al., 2004). However, this review is relatively novel in a few ways. First, it summarizes progress in our understanding of the mechanism and empirical effects of GA within the past couple of decades, since several of the more general reviews on the topic were published. Second, this review discusses the compatibility of GA fixation with a variety of molecular profiling assays, many of which have only recently been invented. Finally, this review focuses on how the biochemical mechanisms of GA fixation can inform how molecular profiling techniques could be improved in the future to study tissue fixed with GA. I hope that this review will be useful to investigators who are considering using GA for banking biological tissue. The review has an emphasis on nervous system tissue and brain banking, which is a particularly common type of tissue banking; however, the principles should be applicable to other tissues as well. It is important to point out that this review is not meant to be exhaustive and discuss all of the available literature on this extensive topic, but rather to highlight key principles that are likely be relevant when GA is used as a part of a tissue banking procedure.

General principles of aldehyde fixation

Brief history of aldehyde fixation

An ideal fixative is generally meant to *fix* – used in the sense of “stop” – molecules, cells, and tissues in their positions at a particular moment of time. This allows investigators to capture a static picture under a microscope and also allows for reproducibility of observations. Investigators interested in freezing, preserving, and/or hardening cells or tissues have employed fixatives for several centuries. The first fixatives used in biology were alcohols, often “spirits of wine,”

which is an archaic term that refers to purified ethanol (Inglehart, 2017). At high concentrations, alcohols act as *denaturing* chemical fixatives that disrupt the tertiary structure of proteins and thereby render them insoluble (Eltoum et al., 2001a). Starting in the mid-1800s, fixatives other than alcohol began to be introduced by scientists interested in the study of cells and tissues. In 1840, Hannover described the use of chromic acid to preserve and facilitate dissection of the brain and spinal cord, pointing out that the preservation quality was retained for months (as cited in (Clarke & O'Malley, 1996)). In 1893, Blum found that formaldehyde (FA) could preserve mouse tissue with only slight shrinkage and distortion of tissues, as compared to alcohol fixed tissues (as cited in (Fox et al., 1985)). In 1963, Sabatini and colleagues introduced GA for use in electron microscopy studies. Their study showed that GA led to the best morphological preservation compared to several alternative aldehydes, including FA and glyoxal, while maintaining at least moderate activity of several enzymes, such as acetylcholinesterase and alkaline phosphatase. The benefits of using GA for the fixation of the CNS in particular, given its high lipid content and soft texture, quickly became widely known (Rewcastle, 1965).

Basic chemistry of glutaraldehyde fixation

This review will not attempt to summarize the vast literature on the chemical reactions involved in GA fixation of biological tissues; readers interested in this topic are directed to other sources (e.g., (Hayat, 1981; Migneault et al., 2004)). However, I will summarize a few basic points. Broadly, GA is thought to act primarily by *crosslinking* biomolecules, primarily proteins, thereby forming a molecular meshwork that locks a diverse set of molecules in place. GA freely crosses cell membranes, and in the cellular environment, GA crosslinks can be formed both within molecules as well as between molecules, with the majority of crosslinks likely occurring within a single molecule and stabilizing its conformation (Subbotin & Chait, 2014). As a result of this crosslinking, GA fixation can have both *direct* biophysical effects on molecules, as well as *indirect* effects on molecules by affecting the milieu in which those molecules exist. Direct effects of GA fixation include changes in location, chemical composition, and molecular geometry (e.g., protein conformation) of molecules. Indirect effects of GA fixation include facilitating or preventing reactions stimulated by enzymes and accelerating or impeding the loss of molecules from the tissue during subsequent steps of the procedure. I will primarily focus on the direct effects of aldehyde fixation because they tend to be more consistent across experiments; however, I will also attempt to highlight important indirect effects of aldehyde fixation that may be relevant during tissue preservation procedures.

The two major aldehydes used to fix biological tissues are GA and FA. FA is the most common choice for most types of tissue banking, including the study of postmortem human brain tissue. FA and GA are frequently used together in fixative mixtures, for example in Karnofsky's fixative (Karnovsky, 1965). GA has a

chemical structure ($\text{HCO}-(\text{CH}_2)_3\text{-CHO}$) that is similar in many ways to that of FA (HCHO). One key difference between GA and FA is that GA contains two aldehyde groups instead of one. The fact that GA is a dialdehyde molecule is thought to account for its more efficient crosslinking abilities compared to FA (Kiernan, 2015). Among other potential reactions, the aldehyde groups can bind with amine, hydroxyl, and thiol groups on reactant molecules to form amine, acetal, and thioacetal bonds, respectively (Marques, Mansur & Mansur, 2013). The more efficient cross-linking ability of GA compared to FA may be especially important in nervous system tissue, because the relatively high lipid content in the nervous system makes it more resistant to fixation (Fix & Garman, 2000). The efficiency of GA crosslinking means that it is able to complete the reaction quickly; for example, in one experiment on different types of purified protein, GA was found to crosslink proteins within 10 seconds (Ouimet et al., 2018). Another key chemical difference between FA and GA is that FA is a smaller molecule and is therefore highly permeable in tissues. At a temperature of 4 °C and concentration of 4%, FA is reported to have a coefficient of diffusion about twice as high as GA (as cited in (Srinivasan, Sedmak & Jewell, 2002)). These differences aside, fixation with GA shares many biochemical properties with fixation by FA, as their aldehyde groups are the major chemical moieties that bind to other molecules. Because FA fixation is more widely used as a fixative for molecular studies, I will occasionally consider the effects of FA fixation as a proxy for the likely effects of GA fixation molecular preservation as well. Notably, paraformaldehyde is a polymerized form of FA, while formalin is an aqueous solution of FA with a small concentration of methanol, both of which will also be considered as types of FA for the purposes of this review.

In aqueous solution, GA takes on a complex mixture of chemical forms depending on the pH, temperature, concentration, time available to reach equilibrium, and presence of other molecules (Migneault et al., 2004). One study using UV absorption and light scattering found that at a GA concentration of 70%, temperature of 20 °C, and pH of 3-8, the majority of GA takes on a cyclic hemiacetal structure, and that approximately 11% of GA aldehyde groups are free (Kawahara et al., 1992). It is possible that polymeric forms of GA can contribute to crosslinking of proteins (Kawahara et al., 1992). In general, the effects of fixation with GA on complex tissues such as nervous system tissue are dependent upon many factors, including the aforementioned factors that affect GA polymerization potential, the choice of buffer, and the presence of other ions such as calcium (Maunsbach & Afzelius, 1999). These ancillary factors need to be considered when evaluating the literature on GA fixation, since the majority of studies choose one set of these parameters, or at most a small number of them, and these choices can be responsible for any differences in GA fixation observed between studies.

Immersion fixation and perfusion fixation

276 The two major methods available for fixing any tissue are immersion
277 fixation and perfusion fixation. Immersion fixation refers to placing the tissue in a
278 bath that includes fixatives and waiting for the fixatives to diffuse into the deep
279 structures of the brain. Advantages of immersion fixation include its relative ease
280 of performance and that it does not rely on an intact neurovasculature system,
281 which may have vessel wall tears and/or be blocked by a clot. Another
282 advantage of immersion fixation is that it allows for different preservation
283 methods to be used on different parts of the tissue after they are cut into tissue
284 blocks. However, immersion fixation causes gradients in fixation quality, wherein
285 the surface layer where the fixation was initially applied has substantially better
286 fixation quality than deeper layers (Maunsbach & Afzelius, 1999; Grinberg et al.,
287 2008). Because immersion fixation relies on diffusion of the aldehyde, and FA
288 has better diffusion than GA, GA is a relatively less effective fixative in studies
289 using immersion fixation.

290
291 Perfusion fixation typically refers to cannulating some part of the existing
292 vasculature system and using a pressure-generating device to drive fixative-
293 containing fluid through the vessels, where it then extravasates out of circulation
294 into the local tissue (Griep & Griep, 2013). In brain banking, the perfusion
295 approach leverages the brain's existing vasculature system, which is so
296 extensive that nearly every neuron in the brain is thought to have its own
297 capillary (as cited in (Spencer & Verma, 2007)). A major advantage of perfusion
298 fixation is that fixative reaches tissue faster and therefore tissue degradation is
299 minimized. There are several important factors to optimize in perfusion fixation,
300 such as hydrostatic pressure, temperature, osmolarity, and pH (Maunsbach &
301 Afzelius, 1999).

302 303 *Antigen retrieval methods in aldehyde-fixed tissue*

304
305 In any discussion of molecular profiling in tissue fixed with aldehydes, it is
306 important to consider the method of antigen retrieval, which attempts to improve
307 antibody staining during immunohistochemistry of aldehyde-fixed tissue. The
308 three major methods for antigen retrieval in aldehyde fixed tissue are enzymatic
309 (e.g., using proteases), chemical (e.g., using reducing agents), and heat-based
310 (e.g., using microwave heating). Protease treatment aims to break down
311 aldehyde crosslinks and thereby enhance immunostaining while attempting to
312 conserve native protein interactions. For example, protease treatment has also
313 been shown to improve the immunoreactivity of γ -aminobutyric acid (GABA) in a
314 dose-dependent manner in GA-fixed rat nervous system tissue (Dobó et al.,
315 1989). In 1978, Weber et al. reported the use of sodium borohydrate, a reducing
316 agent, in GA-fixed mouse cells to improve the visualization of tubulin in
317 immunofluorescence microscopy (Weber, Rathke & Osborn, 1978). Subsequent
318 studies suggested that sodium borohydride may work by reducing the carbon-
319 nitrogen double bonds in GA crosslinks to single bonds, thus allowing for more
320 free rotation and partially restoring the tertiary conformation of protein antigens
321 (Eldred et al., 1983). In 1991, Shi et al. described a method for improving

antigenicity in formalin-fixed, paraffin-embedded tissues by boiling them at temperatures up to 100 °C using microwaves (Shi, Key & Kalra, 1991). A study using a peptide array model system suggests that this temperature-based antigen retrieval method in FA-fixed tissue works by dissociating the intermolecular crosslinks that exert steric hindrance of antibody binding to the linear protein epitope (Bogen, Vani & Sompuram, 2009). Water bath heating has also been shown to increase immunoreactivity in rat brain tissue fixed with a mixture of GA and FA, without significantly affecting ultrastructural morphology (Jiao et al., 1999). While these methods have primarily been applied to the study of proteins in aldehyde-fixed tissue, they are likely to be helpful in the profiling of other molecule types as well.

There are likely physical limits to antigen retrieval in GA fixed tissue. For example, the imine bonds that can be formed when aldehydes such as FA or GA crosslink amines are also found frequently inside of cells in their native state, prior to the addition of any exogenous aldehydes. As one example of many, the enzyme lysyl oxidase catalyzes a reaction that results in the formation of an imine bond (Takaoka et al., 2016). Moreover, aldehydes frequently polymerize prior to crosslinking, meaning that the carbon backbones of the resulting crosslinks can be of variable length. As a result, there is unlikely to be a unique chemical signature that clearly distinguishes aldehyde crosslinks from native imine bonds, which an enzyme could be engineered to naïvely recognize and excise.

Experimental approaches to query the effects of fixation

There are several types of experiments that can be used to query the effects of aldehyde fixation on molecular features. One experiment type includes an aldehyde fixation step and a subsequent attempt to measure a molecule or a set of molecules, such as via binding of a molecular probe (e.g., *in situ* hybridization or immunohistochemistry) or via fragmentation and sequencing. Another experiment type is to label molecules prior to the fixation process with a radioactive atom, and then measure how much of the total radioactive label is retained following the preservation procedure. Radiolabeling experiments tend to be better able to distinguish whether a molecule has been extracted from the tissue as opposed to still present but either sterically inaccessible or slightly shifted in conformation and thus unable to be measured by a molecular probe. Where possible, I will focus on radiolabeling experiments, as they can be more dispositive in this way. However, this is often not possible, because radiolabeling experiments have been performed much less frequently.

Nucleic Acids

Nucleic acids in cells and the nervous system

In the majority of human cells, there are 46 double-stranded DNA molecules made up of approximately 6 billion base pairs. Along with their associated chromatin, these DNA molecules are wrapped into a 3D configuration. As a result of germline differences, somatic recombination, DNA methylation, histone modifications, and accumulation of long-lived RNA molecules, variability in nucleic acids can contribute substantially to long-term variability in the gene expression and function of cells (McKinnon, 2017). For example, olfactory neurons express only one type of olfactory receptor as a result of alterations in their chromosome organization (Monahan, Horta & Lomvardas, 2019). One study using single-cell RNA sequencing in a lymphoblastoid cell line suggests that there are between 50,000 and 300,000 messenger RNA (mRNA) transcripts per cell (Marinov et al., 2014). Consistent with this high intra-cell type variability, transcription appears to occur in bursts. The total number of mRNA molecules in a cell at a given time may vary based on metabolism, electrophysiological activity, circadian state, and many other factors. Once synthesized, RNA is best known for its role in generating proteins, which in turn serve as a major building block of cells. However, RNA molecules serve a wide variety of roles in the cell other than producing protein molecules, including regulating transcription and performing cell-cell communication. Overall, DNA and RNA act to orchestrate the function of cells in the tissues including the nervous system, and as such their preservation and accessibility is a key consideration for any tissue or brain banking method.

Chemical principles of glutaraldehyde fixation of nucleic acids

In most circumstances, GA is thought to preserve nucleic acids *indirectly* through binding to proteins that are in turn bound to nucleic acids and/or creating a crosslinked protein meshwork that captures local nucleic acids. One study mixed excess GA and either isolated bovine thymus DNA or yeast RNA in vitro and measured the reactions via the ultraviolet absorbance at 260 nm (Hopwood, 1975). This study found that GA does not react with either DNA or RNA at room temperature, but begins to have reactions at higher temperatures, including reactions with RNA at 45 °C and with DNA at 64 °C (Hopwood, 1975). The mechanism behind the temperature dependence is likely that hydrogen bonds among nucleic acids are broken at higher temperatures, which allows GA to interact with the revealed nitrogen atoms on the nucleic acid base (Hopwood, 1975). On the other hand, FA has been reported to react with nucleic acids at room temperature in several different ways, including an addition reaction to form a hydroxymethyl group, although these reactions may be prevented if the nucleic acids have extensive secondary structure, such as in single stranded RNA (Srinivasan, Sedmak & Jewell, 2002).

Empirical effects of glutaraldehyde fixation on DNA

Many investigators have used GA, either in a fixative mixture alongside FA or by itself, to preserve DNA molecules for a variety of downstream studies.

413 However, GA is a strong fixative, and harsh extraction methods such as protease
414 treatment are sometimes necessary to facilitate the release of nucleic acids that
415 are trapped within cross-linked protein and DNA-protein complexes. Notably, one
416 study on algae found that while GA preserved DNA content with few or no
417 induced mutations, extraction with proteinase K was unsuccessful and physical
418 grinding in liquid nitrogen was required in order to extract and amplify the DNA
419 content (Xia et al., 2013). Another potential downside of using GA fixation in DNA
420 hybridization experiments is that GA fixation can produce autofluorescence,
421 which can make it more difficult to identify true DNA hybridization signals (as
422 cited in (Bolland et al., 2013)). Only a few studies have directly compared
423 preservation via GA fixation to other methods for preserving DNA and DNA-
424 associated changes. Consistent with the idea that GA does not directly bind to
425 DNA at ambient temperatures, one comparison of DNA preservation methods in
426 plant and fungi tissue found that fixation with 1% GA induced fewer DNA base
427 pair mutations and DNA damage than 3.7% FA (Douglas & Rogers, 1998).

428
429 *In vivo*, very long DNA molecules are frequently wrapped into chromatin,
430 which is composed of a heterogeneous complex of DNA, RNA, and proteins. In
431 general, chromatin structures tend to be well preserved by GA, and GA is a
432 commonly used fixative for studies of chromatin, such as histone post-
433 translational modifications (Houben et al., 2005) as well as studies using
434 chromatin immunoprecipitation-sequencing (Ji et al., 2013). On the ultrastructural
435 level, it has been found that there are no morphological differences in chromatin
436 structure between samples cryopreserved in the vitreous state and samples
437 preserved with GA fixation (Fussner et al., 2012; Ou et al., 2017). In comparison
438 to FA, GA fixation has been found to lead to better preservation of RNA-DNA-
439 protein interactions, at the cost of higher background signal due to the extensive
440 crosslinking of GA (Chu et al., 2011; Ji et al., 2013). Because GA fixation can
441 lead to difficulty in molecular extraction for subsequent assays, GA fixation
442 followed by proteinase K treatment has also been used in chromatin precipitation
443 studies (Rossetto et al., 2013). Taken together, GA fixation tends to preserve
444 chromatin structure for most applications without inducing major artifacts,
445 although it can create a background signal, and extracting the chromatin content
446 for subsequent studies can pose challenges.

447 *Empirical effects of glutaraldehyde fixation on RNA*

448
449
450 Because the quantitative study of RNA content is so critical in biology,
451 several studies have incorporated radiolabeled uridine, which is present in RNA
452 but not DNA, into animals prior to fixation to study the preservation of RNA. One
453 such study measured the amount of radiolabeled uridine retained in sea urchin
454 embryos following GA fixation (Angerer & Angerer, 1981). These investigators
455 found that the retention of RNA was 5.5-fold higher in embryos fixed with GA
456 than in embryos fixed with Carnoy's solution, which is made of a 3:1 mix of
457 ethanol and acetic acid. This same study found that *in situ* hybridization signal
458 increases substantially in the GA fixed tissue following proteinase K treatment,

and that the increase in hybridization signal was proportional to the concentration of proteinase K used. Another study used radiolabeled uridine to measure RNA extraction during fixation of bacterial cells (Graham & Beveridge, 1990). This study found that radiolabeled uridine losses during fixation in 4% glutaraldehyde solution and subsequent washing steps were negligible, while anywhere from 2-5% of the radiolabeled uridine was extracted by a subsequent dehydration step in acetone or ethanol. Finally, one study measured the retention of radiolabeled uridine following fixation of 2.5% GA in rat small intestine tissue sections, and found that GA was as or more effective at insolubilizing the radiolabeled uridine compared with 10% trichloroacetic acid or 0.3 N perchloric acid (Uddin, Altmann & Leblond, 1984). Further, this study found that GA fixation preserved the expected RNA distribution pattern: the majority of radiolabeled uridine was localized in the nucleus, as expected because this is the location of RNA synthesis. Taken together, GA fixation has been found to preserve the location of the majority of RNA molecules in mammalian tissues including the nervous system. However, it does not prevent all loss of RNA in subsequent dehydration steps, and it can make extraction and isolation of RNA more challenging.

Many studies have used GA fixation to preserve tissue morphology when measuring the location of RNA molecules using *in situ* hybridization approaches. Using GA in combination with FA has been reported to decrease tissue deterioration and thereby increase morphologic preservation (Cubas-Nuñez et al., 2017). However, several studies have found that using GA as a fixative also leads to a decrease in hybridization signals, especially at higher concentrations of GA. For example, one study measured hybridization signals from 28S ribosomal RNA in mammalian cells fixed with various concentrations of FA and/or GA, using an average probe size of 100-400 base pairs (Macville et al., 1995). The authors found that increasing the GA dose led to a decrease in the hybridization signal that was partially but not fully ameliorated by use of the protease pepsin. Another *in situ* hybridization study on cerebral ganglia from snails, which used radiolabeled probes with fragment size of 100-500 base pairs, found that adding 0.1% GA to the FA fixative led to improved morphology but decreased hybridization signal in fixed and embedded tissue (Dirks et al., 1992). However, in 60-80 nm thin cryosections, these authors did not identify major differences in the hybridization signal based on the presence or absence of GA in the fixative. A separate group reported that FA combined with 0.1% GA was consistent with good hybridization signal in mammalian brain and endocrine tissue, while the addition of 1% GA abolished the signal (Bloch et al., 1986). Finally, another group reported that radiolabeled hybridization signal was higher in tissue *Drosophila* ovaries fixed with 4% FA and 0.1% GA than pure FA, but that tissue fixed in 4% GA had around a 60% lower hybridization signal than FA (Binder et al., 1986).

Several studies have found that by optimizing protease digestion time and probe size, GA fixation can have advantages over FA fixation. One study on rat retina tissue found that fixation with GA was superior to FA for producing

hybridization signals, because it was easier to optimize the time of proteinase K digestion on GA-fixed tissue and thereby achieve intense mRNA signals spread across the retina (Uehara et al., 1993). These results are consistent with other studies showing or reporting that proteinase K treatment led to dramatically improved hybridization signals in cells fixed with GA (Angerer & Angerer, 1981; Pickering, 1988, p. 45). Another study found that fixative mixtures including GA led to better morphologic preservation as well as slightly higher hybridization signal on virus-infected cell cultures, but only if shorter probe lengths with a mean of 70 base pairs were used (Moench et al., 1985). In this study, even probe lengths of 140 base pairs led to dramatically worse hybridization signals for tissue fixed with GA.

Overall, these results suggest that GA fixation tends to lead to high quality morphologic preservation and RNA retention at the expense of decreased penetration and/or accessibility of RNA molecules, which can often be overcome by treating tissue with permeabilizing proteases and/or using shorter hybridization probes.

Assays for nucleic acids in glutaraldehyde-fixed cells and tissues

A selection of several studies that have used different types of nucleic acid assays on cells or tissues fixed with GA is presented in **Table 1**, alongside the sample type and the concentration of GA used. Regarding RNA assays that rely on the dissociation of RNA molecules, one study found that GA fixation prevented the extraction of RNA molecules from tissue using standard RNA extraction kits (Bowler et al., 2004). However, other studies using optimized protocols on tissues fixed with mixtures of GA and FA that include protease treatment have allowed for the quantification of microRNA molecules (Siebolts et al., 2009), as well as quantitative RT-PCR *in situ* in the adult mouse brain (Cubas-Núñez et al., 2017). To the best of my knowledge, transcriptome-wide studies on cells or tissues fixed with GA have not been reported. However, transcriptome-wide studies on single cells and tissues fixed with FA have been frequently reported (e.g., (Thomsen et al., 2016; Cao et al., 2018)), suggesting that it may be possible to perform RNA sequencing on GA fixed tissue as well. It may be necessary to perform aggressive dissociation steps on GA fixed tissue in order to access RNA molecules on a transcriptome-wide scale, and the resulting yield of unique RNA molecules profiled may be lower than in FA fixed tissue.

Assay	Sample Type	GA % (w/v)	Readout	Molecule Type(s)	Quality	Notes
Chromatin isolation by RNA purification (Chu et al., 2011)	Human cervical cancer cells	1%	DNA sequence results	RNA, DNA, Chromatin	Able to profile genome-wide lincRNA binding sites	
DNA amplification via PCR (Xia et al., 2013)	Cryptomonad cells	2%	DNA sequence results	DNA	DNA sequence is almost identical to the reference sequence	Requires cryogenic grinding
DNA binding dyes	Human	2.50%	Electron	DNA,	High-resolution 3d	

for EM tomography (Ou et al., 2017)	osteosarcoma cells		microscopy	Chromatin	chromatin ultrastructure	
<i>In situ</i> hybridization for EM (Cmarko & Koberna, 2007)	Human cervical cancer cells	0.25%	Electron microscopy	DNA, RNA	Allows for precise molecular localization	
<i>In situ</i> hybridization for LM (Uehara et al., 1993)	Rat retina tissue	0.5% and 2.5%	Light microscopy	DNA, RNA	Intense mRNA signals throughout the retina	Requires proteinase digestion
<i>In situ</i> RT-PCR of RNA (Cubas-Núñez et al., 2017)	Mouse brain tissue	0.50%	Confocal and electron microscopy	RNA	Detection of specific target mRNA molecules alongside morphologic preservation	
Quantitative DNA PCR (Eckford-Soper & Daugbjerg, 2015)	Algae cells	0.10%	PCR cycle number for fluorescence	DNA	Similar to control for 2/4 of the algae types	Trend of better DNA preservation in algae types with less lipid

Table 1. Representative nucleic acid assays compatible with glutaraldehyde fixation.

GA: Glutaraldehyde; **EM:** Electron microscopy; **LM:** Light microscopy; **PCR:** Polymerase chain reaction; **RT-PCR:** Reverse Transcriptase PCR. **W/v:** Percent weight per volume.

In recent years, there have been several technologies developed that profile a large number of types of RNA molecules in addition to capturing their spatial relationships *in situ* (Ke et al., 2013). These spatial gene expression profiling techniques include spatially-resolved transcript amplicon readout mapping (STARmap) (Wang et al., 2018), *in situ* RNA amplification from aldehyde-fixed tissue such as multiplexed error-robust fluorescence *in situ* hybridization (MERFISH) (Chen et al., 2015), DNA microscopy (Weinstein, Regev & Zhang, 2018), and sequential fluorescence *in situ* hybridization (seqFISH) (Eng et al., 2019). Spatial gene expression techniques have typically employed FA fixation as the preservation procedure. However, fixation with FA may not preserve morphology of the tissue as well as fixation with GA. This, in turn, may limit the precision with which morphologic features can be queried, especially as the spatial resolution of the available techniques improves, e.g. via superresolution microscopy for fluorescence *in situ* hybridization-based approaches. It is unclear whether GA fixation will be compatible with the processing steps necessary for profiling RNA via these or other spatial transcriptomics methods. But given the success of previous RNA *in situ* hybridization approaches on GA fixed tissue, this appears likely to be possible in the future given optimization of the processing steps, such as making the probe sizes smaller.

Proteins

Proteins in cells and the nervous system

Molecules made from building blocks of amino acids can be subdivided into three classes: the amino acids themselves and their metabolic derivatives, such as GABA; peptides, which have been arbitrarily defined as consisting of 50 or fewer amino acids; and proteins, which are in turn defined as containing 50 or more amino acids (Alberts et al., 2002a). For the purposes of this review, all of these will be considered proteins. Proteins can be chemically modified in many ways after translation, tend to fold into particular stereotyped shapes depending on the surrounding milieu, and often coordinate with other protein molecules to form complexes. In addition to GABA, several neurotransmitters are either amino acids themselves, such as glutamate, or derived from them, such as dopamine (derived from phenylalanine) and serotonin (derived from tryptophan). In a mammalian cell, there are approximately 10,000 proteins per mRNA (Li, Bickel & Biggin, 2014) and approximately 2-4 million proteins per cubic micron (Milo, 2013). While neurons have heterogeneous shapes, making it difficult to calculate their volume, individual chemical synapses are often around 1 cubic micron in volume (Milo & Phillips, 2015, sec. "HOW BIG IS A SYNAPSE?"), suggesting that there are on the order of 2-4 million proteins per synapse. Proteins coordinate a vast and diverse set of mechanisms that dictate the function of cells, and are often secreted outside of the cell where they interact with other molecules and cells.

Chemical principles of glutaraldehyde fixation of proteins

Protein chains are thought to be the major chemical reactants of GA within cells. At pH levels of greater than 3, GA primarily reacts with free ϵ -amino groups of lysine residues on proteins, a reaction that is pH-dependent (A. Modenez et al., 2018). GA likely interacts with the amino groups of proteins in part by forming imine crosslinks, and in part by forming secondary amine crosslinks, which are more stable (Kiernan, 2015, p. 29). The amount of crosslinking in a protein has been reported in some studies to be associated with the number of lysine residues; however, this is not a straightforward relationship, and it is likely dependent on the distribution of lysine molecules on the surface of the protein molecule (Hopwood, Allen & McCabe, 1970). GA has also been found to react with other protein residues, including tyrosine, although these residues are thought to be less reactive than lysine residues (Habeeb & Hiramoto, 1968). A major difference between GA and FA is that GA forms more extensive protein-protein crosslinks than FA. The chemical explanation for the difference is not fully known, but is thought to relate to two factors. First, FA forms crosslinks between proteins that contain a single methylene bridge, whereas GA has three methylene groups between its aldehyde groups, thus allowing GA to form variable crosslink bridges across longer distances (Stanly et al., 2016). Second, GA's multiple aldehyde groups means that GA tends to multimerize, which also allows for more variable and distant crosslink bridge formation between proteins (Wong, Jameson & Jameson, 2011). Notably, GA has also been reported to have more efficient crosslinking activity than other dialdehyde molecules such as

glyoxal or succinaldehyde, but the reasons for this are poorly defined (Walt & Agayn, 1994).

It is important to emphasize that GA has an immense possible number of chemical reactions with proteins molecules and that the relative propensity for each of them to occur is not yet known (A. Modenez et al., 2018). In order to convey the diversity of possible reactions between GA and protein molecules, I illustrate three protein-GA reactions that are available in the form of crystal structures from the Protein Data Bank: a lysine-arginine intermolecular crosslink in the barnase enzyme (Salem, Mauguén & Prangé, 2010) (**Figure 1A**), a cysteine-GA hemithioacetal adduct in the petal death protein from the carnation flower (Teplyakov et al., 2005) (**Figure 1B**), and a lysine-lysine intermolecular crosslink by polymerized GA in the lysozyme enzyme (Wine et al., 2007) (**Figure 1C**). These structures illustrate how GA can form crosslinks between protein molecules as a single molecule, multimerize prior to crosslinking, and/or form non-crosslinking adduct bonds with proteins, among many other possible reactions.

Figure 1. Examples of covalent protein-glutaraldehyde interactions from crystallography data. In all sub-figures, red dots represent the oxygen atoms in glutaraldehyde and grey dots represent the carbon atoms. **A:** Space-filling diagram of a single glutaraldehyde molecule bonding between the lysine and arginine residues from the barnase enzyme. **B:** Diagram of an adduct bond formed between the sulfur atom of a cysteine residue in the petal death protein and glutaraldehyde. **C:** Space-filling diagram of a lysine-lysine intermolecular crosslink formed by polymerized glutaraldehyde in the lysosome enzyme. This figure was prepared from Protein Data Bank IDs 3KCH, 1ZLP, and 2HTX. **Glut:** Glutaraldehyde.

Empirical effects of glutaraldehyde fixation on proteins

Consistent with its crosslinking mechanism, GA fixation has been shown to retain the vast majority of proteins in most cells and tissues, including the mammalian nervous system. One study that used immersion fixation of rat liver tissue with 2% GA and measured the percent of protein found in the insoluble fraction, an indirect measure of protein preservation, found that approximately 80% of the protein was rendered insoluble, while approximately 6% diffused into the fixative fluid (Hassell & Hand, 1974). However, these investigators speculated that some of the diffusible fluid may have resulted from mechanical damage during cutting of the tissue block. A separate study compared immersion fixation with GA compared to FA in HeLa cells and found that fixation with FA took more than one hour and allowed for multiple changes in the spatial location of proteins, while fixation with GA took approximately four minutes (Huebinger et al., 2018). This same study found that GA fixation retained the vast majority of cytoplasmic proteins, as indicated by the fact that proteins were not found within the membrane blebs that formed during aldehyde fixation, as they were with FA, glyoxal, or acrolein fixation. In a study demonstrating the capacity of the tissue preservation method SWITCH, which includes GA crosslinking as a critical component, one group found that the GA-based SWITCH method led to the retention of 95-97% of total protein content in mouse brain tissue, which was higher than in tissue fixed with FA (Murray et al., 2015). Notably, in the SWITCH method, GA initially penetrates the brain in acidic buffer with pH of ~3, where GA takes on a chemical form that is highly permeable in tissues and does not form substantial crosslinks. Once the GA has penetrated the tissue, a neutral buffer (pH of ~7) is used to convert GA to the form where crosslinking will begin.

In addition to the total retention of protein content, an important aspect of protein preservation following GA fixation is the retention of antigenicity. When this group measured the conservation of antigenicity following the GA-based SWITCH method to the conservation of antigenicity following FA fixation, they found that their GA-based method retained antigenicity of 95% (86/90) of proteins, compared to 92% (83/90) for the FA-based method. Another study used perfusion fixation of rhesus monkey brains with either 4% FA or 1% FA and 1.25% GA, followed by cryoprotection of tissue blocks up to 150 cc in 10% glycerol and 2% DMSO for long-term storage at -80 °C for many years (Estrada et al., 2017). These investigators then used immunohistochemistry to measure the binding of eight protein antibodies. They found that there were no substantial differences in the effect of fixation type on staining, and that storage time had no effect on the intensity of the immunohistochemistry for the majority of antibodies tested. Notably, when protein immunostaining signal is found to be relatively low in GA-fixed tissue sections, it is often attributed to decreased antibody penetration into the tissue (Mrini et al., 1995). This attribution is consistent with the finding that increasing the concentration of GA used for fixation has been found to decrease the immunoreactivity of antigens (Prasadarao, Tobet & Jungalwala, 1990). As discussed above, antigen retrieval methods can

occasionally be used to rescue antigenicity of proteins in cells or tissues fixed with GA.

Another important feature of protein fixation is maintaining the location of proteins within a tissue in close to their *in vivo* positions. Based on the mechanism of the crosslinking reaction, crosslinking of proteins by GA is not likely to cause *major* changes in protein location, as high concentrations of intermolecular crosslinks rapidly form a meshwork that locks molecules in place. However, the crosslinking process may cause *slight* change in locations, as proteins can be pulled in one direction during the formation of the crosslinked mesh framework. Overall, artifacts related to the non-native distribution of proteins following aldehyde fixation are more likely due to movement of the protein prior to crosslink formation (Huebinger et al., 2018) and/or incomplete immobilization. Regarding migration of the protein from its native location prior to crosslink formation, several mechanisms could cause this, such as agonal or postmortem changes occurring after a cell or tissue is deprived of sustenance but before it is fixed (Schwab et al., 1994). Regarding incomplete immobilization, recent studies have tackled this question. One study found that fixation with FA alone was insufficient to inhibit the mobility of plasma membrane-bound protein molecules, while fixation with combination FA and GA was sufficient for the majority of molecules (Tanaka et al., 2010). A subsequent study extended these results and found that in cells with intact plasma membranes, FA was insufficient to inhibit the mobility of three transmembrane receptors, while the addition of GA allowed for adequate fixation of the receptor proteins and prevention of receptor clustering artifacts (Stanly et al., 2016). GA has also been shown to preserve protein-protein interactions without inducing a substantial amount of interactions that do not occur in the native cellular state (Subbotin & Chait, 2014). However, some amount of artifactual crosslink-induced connections between proteins can occur following GA fixation, and this is a potential outcome of GA fixation that needs to be accounted for (Subbotin & Chait, 2014).

Another important aspect of protein structure and function is the presence of post-translational modifications. To the best of my knowledge, there has been no systematic study on the preservation of protein post-translational modifications following GA fixation. However, there is no clear biochemical rationale to suspect that GA fixation would alter or cause the loss of covalent post-translational modifications. Consistent with this, numerous studies have shown that various post-translational modifications are preserved following GA fixation, including phosphorylation (Sasaki et al., 2015), ubiquitination (Liu, Martone & Hu, 2004), citrullination (Bongartz et al., 2007), acetylation (Tas et al., 2017), and succinylation (Yang et al., 2018, p. 2). In a brief, non-exhaustive literature review, I was not able to find an example of a post-translational modification that is consistently lost following GA fixation.

Empirical effects of glutaraldehyde fixation on protein conformation

Protein conformation refers to the three-dimensional shape of a protein, and includes changes in secondary structure such as alpha helices and beta sheets, tertiary structure such as hydrophobic interactions and disulfide bonds, and quaternary structure such as interactions between distinct polypeptides into complexes. Notably, the conformation of many proteins is in frequent evolution *in vivo* depending on shifts in the local chemical environment. The literature regarding the effect of GA fixation on protein conformation is mixed. Overall, GA has been found to induce alterations in protein secondary and tertiary structure, but these are typically thought to be small (Hayat, 1981; Griffiths, 1993). One early study found that GA fixation did not lead to the denaturation of three proteins studied (Hopwood, Allen & McCabe, 1970). Crosslinking by GA fixation is also commonly used in enzyme engineering to stabilize enzyme activity by preventing natural denaturation processes (Walt & Agayn, 1994; Ritter, Newton & McShane, 2014). GA has also been used to stabilize the structure of prion protein, which is a highly conformationally variable protein (Ford et al., 2002). Recently, GA fixation has even emerged, alongside other crosslinking agents, as one of the most effective ways to *query* protein tertiary structure, by integrating GA fixation with mass spectrometry (Subbotin & Chait, 2014; Leitner, 2016). As another metric of protein conformation, enzyme activity is often preserved following brief GA fixation, such as glucose-6-phosphatase in the adult mammalian nervous system (Cataldo & Broadwell, 1986). One reason that GA may preserve the tertiary structure of most proteins is that amino groups, being hydrophilic, are typically located at the surface of proteins, so the GA-amino group bonds that preferentially form tend to not disrupt the structure-generating intramolecular bonds (Hayat, 1981).

However, other investigators have reported that aldehyde fixation can lead to alterations in the conformation of certain proteins (Schnell et al., 2012). For example, one study found that while GA fixation has a negligible effect on the secondary structure of collagen, it induces a change in the secondary structure of silk from a helical or coiled conformation to beta sheets (Zhu et al., 2017). In a separate study, GA fixation was found to eliminate the 22-29% of the alpha helix structure of different free or membrane-bound proteins (Lenard & Singer, 1968). Another study that found that GA crosslinking affected the conformation of cobrotoxin (Chang, Lin & Yang, 2001). Furthermore, fluorescence of fluorescent proteins, which is dependent on protein conformation, is frequently lost following GA fixation (Seo, Choe & Kim, 2016; Park et al., 2019). One approach to the loss of fluorescence in green fluorescent protein in cells once they are fixed with GA has been to fix them first with FA, image them using the confocal microscope, and then fix the cells with GA to preserve their ultrastructure for electron microscopy (Gil-Perotin et al., 2016). Overall, the effect of GA fixation on protein conformation appears to be variable, likely reflecting a heterogeneous effect depending on the protein, its environment, and the relative concentration of GA.

Empirical effects of glutaraldehyde fixation on protein neurotransmitters

Neurotransmitters have been defined as molecules that are stored in synaptic vesicles, are released by exocytosis, and then bind to a receptor on the target cell (Miyaji et al., 2008). Many neurotransmitters are derived from amino acids, such as the monoamine class of neurotransmitters that includes serotonin, histamine, and dopamine. Using serotonin as an example, studies have reported using GA perfusion fixation for immunofluorescence using an anti-serotonin antibody (Lyons et al., 2016) as well as using GA fixation for immunoelectron microscopy labeling of serotonin using an anti-serotonin antibody (Kiliaan, Scholten & Groot, 1997). On the other hand, a comparative study of quantitative serotonin immunoreactivity in rat midbrain tissue sections found that serotonin immunoreactivity was decreased in samples fixed with combination GA/FA as opposed to FA only (Clements & Beitz, 1985). As with other immunohistochemistry studies using GA, the reason for the decrease in serotonin immunoreactivity is likely that GA fixed tissue causes more steric hindrance for antibody penetration and binding, as a result of its more extensive crosslinking. Notably, GA can lead to better retention of small protein molecules than FA; for example, one study found that perfusion fixation with GA at high concentrations of 2-5% was necessary for the retention of glutamate in rat brain tissue, while FA perfusion fixation was insufficient (Storm-Mathisen & Ottersen, 1990). Peptides, which frequently act as local modulators in nervous systems, will likely follow similar preservation patterns in GA-fixed tissue. For example, one study on spinal cord sections that were perfusion-fixed with 2.5% GA found that cutting the ends of nerve fibers and treating them with ethanol allowed for immunostaining of the neuropeptides neuropeptide Y, substance P, and enkephalin (Llewellyn-Smith & Minson, 1992).

Protein assays on glutaraldehyde-fixed tissue

The major protein assays performed on tissue fixed with GA are in the immunostaining class, either via light microscopy in immunohistochemistry or via electron microscopy in immunoelectron microscopy (**Table 2**). As discussed above and seen in several studies, compared to fixation with other aldehydes, GA tends to have better preservation of *in vivo* spatial locations while having worse penetration of antibodies and accessibility of antigens for binding. It is possible that the expanding use of aptamers to visualize proteins in tissues will make GA a more appealing fixative, as aptamers are generally smaller than antibodies and therefore have better tissue penetration (Bayat et al., 2018). While proteomics can be performed on GA-fixed tissue, the yield is generally much lower, in part because the extensive crosslinking decreases the solubility of proteins, and in part because proteomics methods frequently rely on dissociation of proteins as one of the steps in profiling them. For example, one group compared fixation followed by proteome profiling using FA and either GA or the reversible crosslinker dithiobispropionimidate (DTBP), finding that the yield of unique peptides was around five times higher in cells fixed with DTBP (Gordon, Kannan & Gousset, 2018). However, one advantage that GA offers in proteomics studies is that, since GA leads to rapid cross-linking of protein complexes *in situ*,

assays can leverage GA fixation as a step for studying protein-protein interactions (Ethier et al., 2006; Ouimet et al., 2018). Overall, because proteins are predominantly physically *present* in GA-fixed tissue, just difficult to access, it stands to reason that future technologies may be able to improve the capabilities to profile proteins in tissue banked using GA.

Assay	Sample Type	GA % (weight / volume)	Readout	Molecule Type(s)	Quality	Notes
Immunoelectron microscopy (Hinova-Palova et al., 2014)	Cat claustrum tissue	2.50%	Electron microscopy	Peptide	NPY immunoreactivity observed in ultrastructural images	
Immunohistochemistry (Bombardi et al., 2018)	Rat brain tissue	0.20%	Light microscopy	Amino acid	GABA immunoreactivity observed in neurons and neuropil	
Liquid chromatography-mass spectrometry proteomics (Subbotin & Chait, 2014)	Yeast cells	No %, but GA-to-lysine molar ratio of ~1:5	Mass spectrometry data	Protein	Identifies protein assemblies, not the crosslinked peptides themselves	Cryogenic grinding prior to low-temperature GA crosslinking
Mass spectrometry proteomics (Gordon, Kannan & Gousset, 2018)	Mouse neuronal tumor cells	0.05%	Mass spectrometry data	Peptide	Around 5-fold fewer unique peptides recovered with GA than when using the reversible crosslinker DTBP	Used laser capture microdissection to isolate tissue

Table 2. Representative protein assays compatible with glutaraldehyde fixation. **GA:** Glutaraldehyde; **DTBP:** Dithiobispropionimide; **NPY:** Neuropeptide Y; **GABA:** γ -aminobutyric acid.

Lipids

Lipids in cells and the nervous system

The human brain is a highly lipid-rich organ, and this unique aspect contributes heavily to its distinct biophysical and functional properties. Quantitatively, mouse cortical pyramidal neurons have been found to have an average surface area of approximately 20,000 μm^2 (Rocher et al., 2010). Given an average of approximately 5,000,000 lipid molecules per 1 μm^2 (as reported in (Alberts et al., 2002b)), there are approximately 100 billion lipid molecules per neuronal cell membrane. This does not include lipids in the extracellular or intracellular space including organelle membranes, or lipids in other cell types including oligodendrocytes, whose myelin make up approximately 50% of lipid synthesis in the brain (Smith, 1973). Lipids play important roles in cell signaling in the brain; for example, they have a distinct composition at synapses, where they form distinct domains that house neurotransmitter receptors (Borroni, Vallés & Barrantes, 2016). There are several categories of lipids, and humans have been

found to have a highly distinct lipid composition compared with other mammalian species (Bozek et al., 2015). Consistent with this, alterations in lipid levels have been found in several classes of psychiatric disorders such as depressive and anxiety disorders (Müller et al., 2015). Notably, ultrastructure visible on electron microscopy is not a perfect correlate for lipid content. For example, removing cholesterol does not appear to affect the appearance of cell membranes on electron microscopy (as reported in (Macville et al., 1995)).

Chemical principles of aldehyde fixation of lipids

Lipids are defined as molecules that are insoluble in water but are soluble in organic solvents; that is, they are hydrophobic (Fahy et al., 2011). There are many subclasses of lipids including fatty acids, phospholipids, sterols, and sphingolipids. They are a more complex, heterogeneous set of molecules than nucleic acids, proteins, and carbohydrates, because they are not as easily described in terms of their monomer building blocks. This heterogeneity makes it more difficult to summarize the chemical reactions of aldehyde fixatives with lipids. However, a considerable literature is available on this topic, making it possible to discuss several of the details.

In general, GA is not thought to bind directly to most types of lipids. Instead, the lipids that are preserved following GA fixation are primarily held in place by the creation of crosslinked protein complexes that either capture proteins bound to lipids or otherwise slow or prevent their diffusion. However, there are a few exceptions where GA has been found to bind to some particular lipid types. For example, GA has also been found to have reactivity with phospholipids that do contain free amino groups, such as phosphatidylserine and phosphatidylethanolamine (Russell & Hopwood, 1976). GA has also been reported to bind to sphingolipids via a reversible reaction with their 1,3-diol groups, which can be used to concentrate sphingolipids for downstream analysis (Gowda et al., 2018). Consistent with the lack of direct binding between GA and most lipids, studies have found that plasma membranes tend to bleb when fixed with GA (Huebinger et al., 2018). Furthermore, if tissues or organs fixed with GA or FA are stored at room temperature for long periods of time, the lipids in them will tend to migrate towards either the surface or interior of the tissue or organ, depending on the environment and the polarity of the lipids (Sjövall, Johansson & Lausmaa, 2006). This is one reason why tissue banking methods which exclusively use FA or GA fixatives will not lead to long-term lipid immobilization and retention in the absence of additional steps, such as cryopreservation (Rosene, Roy & Davis, 1986) or additional fixation with osmium tetroxide (Mikula & Denk, 2015), the latter of which fixes unsaturated fatty acids, potentially via crosslinking them (Eltoum et al., 2001b; Belazi et al., 2009).

Empirical effects of glutaraldehyde fixation on lipids

One way to categorize the effects of GA crosslinking on lipid preservation is to distinguish between the direct and indirect effects of GA. Direct effects are perhaps best studied in isolated cell samples, where lipids are less likely to be affected by other factors. On the other hand, indirect effects are perhaps best studied in tissues, where GA is perfused or where the diffusion time of GA is more relevant↓.

The direct effects of GA fixation on lipid preservation in isolated cell samples tend to be minimal. For example, in one experiment in which the effect of GA fixation on multiple myeloma cells was queried by mass spectrometry, GA fixation did not affect the normalized mean intensity of five types of fatty acids compared with unfixed cells (Nagata et al., 2014). Another study compared the effects of GA immersion fixation on lipid content in mesenchymal stem cells compared with several other sample preparation methods (Schaepe et al., 2015). In this study, GA and FA fixation led to similar lipid preservation profiles, and, in contrast to alcohols, GA and FA not decrease the content of phospholipids (the main constituents of cell membranes) or phosphatidylcholines. In the mesenchymal stem cells studied, fixation with GA and FA were also found to have increased lipid preservation content and reproducibility compared with freeze-drying, freeze-fracturing, and cryofixation. Consistent with the lack of direct binding between GA and most lipids, another study found that fixation of human T24 cells with 0.2% GA immobilized glycosylphosphatidylinositol-anchored proteins but not phospholipids or cholesterol (Tanaka et al., 2010).

One study compared the effects of GA fixation and freeze drying of fibroblast cells with ammonium formate washing followed by cryofixation and freeze drying (Malm et al., 2009). In this study, both methods were found to retain lipid distributions with similar accuracy. However, the GA fixation method appeared to have superior preservation of the smallest lipid structures, while it appeared to also lead to a larger decrease in cell membrane integrity. A combination GA and FA immersion fixation method has also been used to successfully study fatty acid content using mass spectrometry in the differentiation pathway of cultured adipocytes and osteocytes (Schaepe et al., 2017). Finally, another study in lymphatic endothelial cells found that a combination GA and FA fixative, but not FA alone, was able to prevent membrane lipid diffusion after fixation, thus better preserving the relative location of membrane lipids (Stanly et al., 2016).

The direct and indirect effects of GA immersion fixation in tissues on lipid preservation have also been studied by sequential approaches, which measure the extraction of lipids at various points in a tissue processing procedure. One study incubated radiolabeled acetate in rat brain tissue as a proxy for lipid content and measured extraction of the radiolabeled lipids at each step in the processing procedure (Maneta-Peyret et al., 1999). The initial GA immersion fixation step led to an extraction of around 2-12% of the lipid content from the brain tissue. The buffer wash step immediately after GA immersion fixation led to

losses of 20-25% of lipids, and subsequent ethanol dehydration steps also led to substantial lipid extraction. Total lipid losses in the study reached an average of 73-91%, depending on the precise protocol used, suggesting that GA immersion fixation did not bind the lipids strongly enough to prevent subsequent extraction by harsh processing steps such as dehydration. Another study measured the amount of radiolabeled lipids lost during GA immersion fixation and subsequent processing steps in rat liver tissue samples (Leist, Nettleton & Feldhoff, 1986). They found that only 0.5% of the lipids were lost during the GA immersion fixation step. Higher levels of lipid losses occurred during the ethanol dehydration step, including 25% of lipids extracted from tissue fixed in GA and 62% of lipids extracted from tissue fixed in FA. Finally, another study measured the extraction of radiolabeled lipids in bacterial cells following fixation with 1% GA at 4 °C. This study found that less than 1% of the lipids were extracted during the GA fixation step (Weibull, Christiansson & Carlemalm, 1983).

In addition to radiolabeling, one approach is to measure lipids directly at each step of the preservation procedure. One study used this approach with thin-layer chromatography on frog retinas fixed with either GA or FA (Nir & Hall, 1974). This study found that the initial fixation process extracted less than 0.5% of the total lipid content with either GA or FA. The authors then studied how much GA and FA fixation prevented subsequent extraction by chloroform-methanol treatment. They found that in GA-fixed tissue, 38% of the phospholipids were retained in the tissue after chloroform-methanol treatment, as compared to only 7% in FA-fixed tissue. Another study used a similar approach with thin-layer chromatography to show that subsequent to both GA and FA fixation, a large proportion of lipids are extracted by chloroform-methanol from rat hippocampus tissue (Roozmond, 1969). The lipid types that were retained in the rat hippocampus tissue were phosphatidylserine and phosphatidylethanolamine, which was a much stronger effect for GA than for FA. The authors of both of these studies speculate that GA fixation may have resulted in less lipid extraction compared to FA fixation because of increased direct binding between GA and phosphatidylserine and phosphatidylethanolamine. Consistent with this, another study evaluated the effect of GA fixation on myelin constituents found that phosphatidylserine and phosphatidylethanolamine were specifically retained by GA fixation after chloroform-methanol extraction (Wood, 1973).

Because many brain banking methods perform the initial GA fixation step via perfusion, an especially important experimental modality to consider is the effect of perfusion fixation using GA on lipid levels. One study evaluated the effect of perfusion fixation with GA on brain lipids in mice, rats, and rabbits (Levy et al., 1965). These investigators found that perfusion fixation with GA did not cause significant changes in the levels of the brain lipid classes that they measured, including total lipids, cholesterol, or phosphatides. While not a pure GA fixation study, another study used radiolabeling to measure lipid extraction following combination GA and carbonylhydrazide perfusion fixation in cardiac tissue (Ward & Gloster, 1976). These investigators found that their combination GA and

carbohydrazide fixation method allowed for the retention of 82% of lipids following chloroform-methanol treatment.

A particularly important class of lipids with respect to brain banking is steroid hormones, for which the evidence of retention during GA fixation is mixed. One review noted that radiolabeled steroid hormones are generally lost following fixation with aldehydes such as GA, which may be related to their high diffusibility (Bullock, 1984). Another study found that immersion fixation of human placental tissue with 2.5% GA for 8 to 12 hours led to less leakage of radiolabeled steroid hormone compared to FA and other fixatives, although it still allowed for the release of 50% of the placental steroid hormone content from the tissue into the fixative solution (Dobashi et al., 1985).

Overall, while there is some variation between studies that can be attributed to differences in experimental design, a general conclusion from these studies is that GA fixation itself leads to minimal direct extraction or loss of most lipids. However, GA fixation, while superior in this regard to no fixation or FA fixation, is not able to prevent the extraction of a substantial amount of lipids by diffusion or by subsequent dehydration steps. An exception to this tendency is for some specific lipid species such as phosphatidylserine and phosphatidylethanolamine, which have been found to be retained in samples preserved by GA fixation even after dehydration.

Lipid assays in glutaraldehyde-fixed tissue

Cells and tissues fixed with GA are compatible with several different lipid dyes and other assays (**Table 3**). For example, one study that used GA fixation to preserve white matter regions in cats and then luxol-fast blue stains found that the staining intensity was able to distinguish decreased myelin sheath density in cats with a genetic myelin deficiency (Vite et al., 2001). As another example, one study used Nile red to stain for lipid droplets in GA-fixed peritoneal macrophages, finding that there was no apparent difference in fluorescence intensity or distribution between fixed compared to unfixed cells (Greenspan, Mayer & Fowler, 1985).

Assay	Sample Type	GA % (w/v)	Readout	Molecule Type(s)	Quality	Notes
Immunocytochemistry with LM and EM (Hirono et al., 2004)	Dissociated frog hair cells	0.75%	Light microscopy, electron microscopy	Lipids	PIP2 immunoreactivity seen with confocal LM images or gold-labeled EM images	Some cells may have been permeabilized with 0.2% saponin
Luxol fast blue stain for myelin (Vite et al., 2001)	Cat white matter regions	1.30%	Light microscopy	Myelin sheath	Staining intensity can distinguish myelin sheath density in different groups	
MALDI MS imaging	Human	0.25%	Mass	Lipids	Lipid spatial	Cells grown on

on lipid spatial distribution in single cells (Schober et al., 2012)	cervical cancer cells		spectrometry data		distribution profiles from 7 μ m pixels	glass slides
Nile red stain for lipid droplets (Greenspan, Mayer & Fowler, 1985)	Mouse peritoneal macrophages	1.50%	Light microscopy	Lipid droplets	No apparent differences in fluorescence between GA-fixed and unfixed cells	
Time-of-flight secondary ion mass spectrometry imaging (Nagata et al., 2014)	Human multiple myeloma cells	0.25%	Mass spectrometry data	Lipids	No significant differences compared to unfixed cells	Cells were fixed on a glass slide, and rinsed in ammonium acetate solution prior to mass spectrometry

Table 3. Representative lipid assays compatible with glutaraldehyde fixation. **GA:** Glutaraldehyde; **MS:** Mass spectrometry; **MALDI:** Matrix Assisted Laser Desorption/Ionization; **LM:** Light Microscopy; **EM:** Electron microscopy; **PIP2:** Phosphatidylinositol 4,5-bisphosphate. **W/v:** Percent weight per volume.

While mass spectrometry analysis of lipids tends to be used in frozen tissue or frozen tissue powder (Bozek et al., 2015), it can also be performed in tissue fixed with GA. For example, one study used time-of-flight secondary ion-mass spectrometry to profile lipids in GA-fixed multiple myeloma cells, finding no difference in lipid profiles between fixed and unfixed cells (Nagata et al., 2014). Another group used matrix-assisted laser desorption ionization imaging mass spectrometry to visualize the spatial distribution of lipids in cervical cancer cells fixed with GA, from which they were able to detect lipids at a resolution of 7 μ m (Schober et al., 2012).

Finally, it is also possible to do immunocytochemistry or immunohistochemistry to visualize lipids that are retained in tissue fixed with GA. For example, one study used antibodies against the cell membrane phospholipid phosphatidylinositol 4,5-bisphosphate in dissociated frog hair cells and visualized the resulting immunoreactivity with both confocal microscopy and electron microscopy (Hirono et al., 2004). One possible concern here is that GA fixation does produce autofluorescence that is particularly strong in myelin and can decrease the signal to noise ratio for antibody fluorescence. Notably, this myelin autofluorescence can also be exploited for myelin imaging (Christensen et al., 2014).

Carbohydrates

Carbohydrates in cells and the nervous system

Carbohydrate polymers can be classified into storage polysaccharides, which provide energy upon their breakdown, and structural polysaccharides, which form long-lasting components of cells and tissues molecules. The major storage polysaccharide in humans is glycogen, which is broken down to the

monosaccharide glucose. Glucose is the major energy supply for the brain during most conditions, although in certain circumstances such as fasting, ketone bodies can sustain the brain's energy requirements (Magistretti & Allaman, 2015). The brain uses a disproportionately large amount of energy relative to its dry mass, and the majority of this energy is likely used in sustaining the numerous ionic membrane potentials required for electrochemical communication (Berg, Tymoczko & Stryer, 2002). Astrocytes have been found to play a particularly important role in brain metabolism because of their unique ability to store glycogen, which can be broken down via glycolysis into lactate and pyruvate and shuttled to neurons (Magistretti & Allaman, 2015). In animals, an important class of structural polysaccharides are the glycosaminoglycans, which includes heparan sulfate, chondroitin sulfate, and hyaluronan. Hyaluronan makes up a major component of the extracellular matrix and is particularly abundant in the nervous system, where it forms a scaffold for perineuronal nets (Hascall & Esko, 2015). In addition to polysaccharides, freestanding carbohydrate molecules are also found in the cytoplasm, where they can act as regulatory switches (Varki & Kornfeld, 2015). One of the other major roles for carbohydrates in mammalian cells is to covalently bond with proteins and lipids to form glycoproteins and glycolipids, which can endow proteins and lipids with unique properties.

Chemical principles of glutaraldehyde fixation of carbohydrates

In general, GA is thought to fix carbohydrates primarily via crosslinking the amino acid residues of glycoproteins and/or trapping freestanding carbohydrates in the GA crosslinked meshwork. However, GA is also able to bind to some carbohydrate molecules directly, for example by binding to amino groups to form imine bonds or binding to hydroxyl groups to form acetal bonds (Campos, Coimbra & Gil, 2012). For example, GA has been shown to crosslink the carbohydrate starch via hemiacetal bonds (Gonenc & Us, 2019) and to crosslink hyaluronan under acidic conditions, which is believed to either be with a hemiacetal or ether bond (Collins & Birkinshaw, 2013). It has also been described that GA may have reactivity under certain circumstances with glycogen and mucopolysaccharides (Hayat, 1981, p. 90), likely through forming acetal bonds with their hydroxyl groups. Notably, imine bonds are generally considered more stable than acetal bonds, as acetal bonds are more likely to be hydrolyzed (Olde Damink et al., 1995). Carbohydrates less frequently have amino groups that can react to form the relatively more stable imine bonds, which may help explain why GA tends to have less chemical reactivity with carbohydrates than it does with proteins.

Empirical effects of glutaraldehyde fixation on carbohydrates

Studies have shown that GA fixation tends to retain a substantial percentage of the major storage polysaccharide in mammalian tissues, glycogen. One study on mouse CNS tissue found that the expected glycogen distribution

patterns were retained following perfusion fixation with 2% GA/2% FA (Cataldo & Broadwell, 1986). This study used electron microscopy to visualize the staining patterns of glycogen. As a negative control, they also showed that digestion with 0.1% malt diastase after perfusion fixation with GA but before postfixation in osmium tetroxide eliminated all glycogen staining. One study measured glycogen and glycogen-associated proteins in rat liver tissue immersion fixed with either the "weak fixation" protocol of 1% GA or the "strong fixation" protocol of 5% GA and 3% FA, followed by post-fixation with 1% osmium tetroxide and a saturated solution of lead citrate (Bendayan et al., 2009). After the fixation protocols, the tissue was dehydrated in an alcohol and embedded. This group found that glycogen and the glycogen-associated protein AMP-activated protein kinase was only found in the electron micrographs of tissue that had been preserved in the "strong fixative," suggesting that at least one of the 5% GA, 3% FA, or their combination was necessary for retaining the glycogen after the tissue is dehydrated. Notably, one other study found that GA tended to fix more glycogen content than FA in biopsy liver tissue (McAdams & Wilson, 1966). Quantitatively, one author reported that approximately 40-63% of glycogen is retained following GA fixation in liver or heart tissue (Hayat, 1981). Another group found that approximately 50% of radiolabeled 2-deoxyglucose was preserved following GA fixation and dehydration in invertebrate ganglia, noting that some of the label may have been incorporated into glycogen, and that there is variability in other studies (Kai Kai & Pentreath, 1981). Glycogen levels have been found to decrease rapidly during ischemia, and have been found to virtually disappear after 10-20 minutes of ischemia (Lowry et al., 1964). Consistent with this, another author reported that ischemia- or hypoxia-associated changes in tissues prior to the fixatives entering cells are often responsible for glycogen losses (Bullock, 1984). Overall, it is unclear the degree to which glycogen is quantitatively retained by GA fixation alone, and this likely depends on several factors specific to each experiment, including the concentration of GA used and the amount of ischemia that the cells experience prior to fixation. In addition to glycogen, evidence has shown that GA fixation tends to retain most complex carbohydrates (as cited in (Thomopoulos, Schulte & Spicer, 1987)).

Empirical studies of the effects of GA fixation on structural polysaccharides have also tended to show that they are generally retained by the procedure. For example, one study found that GA fixation preserved the secreted extracellular polysaccharide envelope of gram-negative bacteria for visualization using scanning electron microscopy (Politis & Goodman, 1980). On the other hand, the literature on retention of hyaluronan following GA fixation is mixed. One study reported that rat cerebellar tissue perfusion fixed with 2%FA/2% GA as well as 0.5% of the quaternary ammonium compound cetylpyridinium chloride preserved hyaluronan such that it was able to be stained by a biotinylated hyaluronan-binding complex (Ripellino et al., 1985). As negative controls, this study showed that the staining was obliterated by treatment of the tissue with hyaluronan oligosaccharides or with hyaluronidase. When the authors removed cetylpyridinium chloride from the perfusate, hyaluronan staining was not visible,

and therefore the authors suggested that hyaluronan might not be immobilized by aldehyde fixation alone for retention following dehydration. Another group of investigators reported that hyaluronan staining was present in rat peripheral nerve fascicles fixed with GA and microwave irradiation, followed by cryoprotection, freezing, freeze substitution, and embedding in epoxy resin (Eggli et al., 1992). They speculated that their use of microwave irradiation reduced the required fixation time and thus decreased extraction of the highly water-soluble hyaluronan, although they did not directly report on GA fixation alone compared to GA fixation with the addition of microwave irradiation. Another study used GA immersion fixation and osmium tetroxide postfixation on cerebellar tissue blocks to identify electron dense material in synapses that was sensitive to hyaluronidase (Castejón & Castejón, 1976). However, inconsistent with this, other studies have reported successful fixation of hyaluronan using aldehyde fixatives alone. One study reported that perfusion fixation with 4% FA/0.1% GA of mouse hippocampus allowed for the retention of hyaluronan in the extracellular matrix following alcohol dehydration and embedding (Brückner et al., 2003). Another study used GA immersion fixation alone and alcian blue staining of the vitreous base of the mouse eye to study carbohydrates (Rhodes, 1985). This study found that a globular material, which was thought to be hyaluronan because it was digested by hyaluronidase, was visualized following the GA fixation procedure. Because at least one study shows that aldehyde fixatives alone can retain hyaluronan during perfusion fixation of brain tissue, it is likely that there is a quantitative extraction of hyaluronan that depends on other factors related to the experiment, including the speed and quality of fixation.

Carbohydrate assays in glutaraldehyde-fixed tissue

It is possible to study many types of carbohydrates in GA fixed tissue with molecular probes that are specific for that carbohydrate (**Table 4**). One example assay is using the biotinylated probe used for hyaluronan (Ripellino et al., 1985). Several microscopy techniques are also capable of detecting carbohydrates, such as the use of atomic force microscopy to measure surface carbohydrates in bacteria (Wang, Ehrhardt & Yadavalli, 2015). In addition to the alcian blue stain (Rhodes, 1985), one important stain that is frequently used to measure polysaccharides is the periodic acid–Schiff stain. This stain relies on breaking up polysaccharides into monosaccharide units and thereby creating free aldehyde groups that can react with the Schiff reagent. Because GA fixation also leads to the presence of free aldehyde groups, GA can induce false positives when using the periodic acid–Schiff stain (Bullock, 1984). However, is thought to be a concentration dependent effect. For example, one study found that rat kidney tissue fixed in 2% GA had a magenta background that more appreciably interfered with periodic acid–Schiff staining, while tissue fixed in 1% GA did not mask Schiff reagent positivity (McDowell & Trump, 1976).

Assay	Sample Type	GA % (weight / volume)	Readout	Molecule Type(s)	Quality	Notes
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Alcian blue staining for hyaluronan (Rhodes, 1985)	Mouse vitreous base	4%	Electron microscopy	Structural polysaccharide	Contraction of hyaluronan content compared to fixative that included cetylpyridinium chloride	
Atomic force microscopy of surface carbohydrates (Wang, Ehrhardt & Yadavalli, 2015)	Bacillus cereus cells	2%	Force profiles	Carbohydrates	Specific carbohydrates were detected at the nanoscale level across the entire cell	Samples were immersed in ethanolamine solution to block GA in the areas without cells
Immuno-EM microscopy of glycogen via staining with reduced osmium tetroxide (Cataldo & Broadwell, 1986)	Mouse brain tissue	2%	Electron microscopy	Storage polysaccharide	Intracellular glycogen particles are most abundant in astrocytes and sparse in neurons	
Periodic acid–Schiff stain for glycogen (McDowell & Trump, 1976)	Rat kidney tissue	1%	Light microscopy	Storage polysaccharide	Schiff reagent positivity is able to be visualized despite a faint magenta background	

Table 4. Representative carbohydrate assays compatible with glutaraldehyde fixation.

GA: Glutaraldehyde; **EM:** Electron microscopy.

Conclusions

It is possible to summarize the chemical principles and empirical effects of GA fixation across the four molecule types considered in this review as follows (**Figure 2**). GA primarily crosslinks proteins, which is likely as a result of their many available amino groups, and as a result forms a system of extensively crosslinked protein molecules. This leads to high-fidelity spatial preservation of protein content. As a result of these crosslinks, GA occasionally leads to chemical changes in the secondary and tertiary structure of proteins, and frequently diminishes their accessibility via molecular probes. There are exceptional cases where GA binds directly to macromolecules other than proteins, such as phosphatidylserine, phosphatidylethanolamine, or certain polysaccharides. But in general, GA fixation leads to the spatial preservation of nucleic acids, lipids, and carbohydrates as a result of their indirect interactions with proteins and via steric effects that limit their movement out of cells and tissues. Harsh downstream processing steps such as dehydration frequently extract members of the non-protein macromolecule classes, thus destroying their spatial preservation, although this can be mitigated by using a high concentration of GA or by using an additional class of fixative such as osmium tetroxide.

Figure 2. Summary of the effects of glutaraldehyde fixation on the four major classes of macromolecules.

While proteins are directly crosslinked by glutaraldehyde, the glutaraldehyde crosslink mesh that forms indirectly retains many or most nucleic acids, lipids, and carbohydrates. However, these latter macromolecular classes can also be frequently lost following downstream processing steps such as dehydration. Protein tertiary structure seems to be more commonly altered than secondary structure following GA fixation, and this can lead to the loss of structural properties, such as fluorescence. **GA:** Glutaraldehyde; **Protein 2°:** Protein secondary structure; **Protein 3°:** Protein tertiary structure.

As noted in the introduction of this review, there is a trade-off between tissue fixation approaches regarding their subsequent effects on morphology and molecular assays. This trade-off exists within GA fixative concentrations as well, as higher concentrations (such as 4% weight/volume) generally lead to improved outcomes in morphology studies, but have decreased performance in many molecular assays. Regarding GA fixation, it is likely that this trade-off is due to both limitations in the currently available assays, which may improve with improved antigen retrieval and creative dissociation techniques, as well as

fundamental biology, such as the formation of imine bonds that are difficult to distinguish between artifactual crosslinks and native proteins. It is possible to decompose the effects of higher concentrations of GA on molecular assays into more precise terms. First, several studies have found that higher GA concentration leads to improved *retention* of molecules within the tissue, especially molecules that are poorly fixed by GA, such as glycogen. This is likely due to a more extensive crosslink meshwork that traps more molecules and/or the effect of more free aldehydes to perform less energetically favorable acetal bond formation with hydroxyl groups. Second, higher GA concentrations likely lead to more *alterations* of individual molecular structures, such as protein conformation, as more structure-disrupting intramolecular bonds are formed. The effects of higher GA concentration on protein conformation will also inhibit several molecular assays such as enzyme activity studies. Titrating the GA concentration to find the optimal balance of molecular effects depends on the goals of the investigator.

When evaluating GA fixation for use in tissue preservation techniques such as brain banking, it is helpful to need to think about what studies will be performed on the tissue. Historically, the majority of molecular assays have been performed on frozen tissue, while fixed tissue has been limited primarily to *in situ* hybridization and immunohistochemistry (Schmitt et al., 2007). However, improvements in assays may allow for a wealth of studies including aptamer staining for proteins (Gomes, Höbartner & Opazo, 2017), spatial transcriptomics studies for nucleic acids, and mass spectrometry imaging for spatial metabolite distribution studies of lipids, nucleic acids, and carbohydrates (Schober et al., 2012). From a tissue banking perspective, fixation with an aldehyde such as GA offers the important advantage of improved retention of tissue morphology, which allows for high-resolution cell-cell connectivity studies such as connectomics. While cryopreservation can also achieve this goal, cryopreservation is either limited to very thin tissue slices in the absence of crosslinking molecules (Frotscher et al., 2014) or requires initial fixation with aldehydes in order to stabilize tissue and prevent dehydration during cryoprotection (Meissner & Schwarz, 1990; McIntyre & Fahy, 2015). An alternative approach would be to use a reversible crosslinker such as DTBP, which might allow for ultrastructure preservation similar to GA while also allowing for improved molecular resolution in certain assays such as proteomics (Gordon, Kannan & Gousset, 2018). Because of the ubiquity of fixation in pathology and the advantages of fixed tissue in tissue architecture preservation, it is likely that molecular assays on fixed tissue will continue to be an active area of technology development in the upcoming years. With regards to brain banking, it is plausible that tissue banking techniques and molecular assays will develop such that it makes sense in many cases to fix the entire brain rather than just one hemisphere, which would allow for improved perfusion quality and the ability to interrogate hemispheric laterality.

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