Effects of Preanalytical Variables on the Detection of Proteins by Immunohistochemistry in Formalin-Fixed, Paraffin-Embedded Tissue

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RATIONALE

Routine, formalin-fixed, paraffin-embedded (FFPE) tissue specimens are analyzed by immunohistochemistry within clinical and research laboratories around the world. However, this “routine” process that was developed decades ago is one that is both complex and variable. Marked differences in specimen-processing regimens are prevalent both within and among institutions owing to a lack of standardization and formal standard operating procedures. Thus, the influence of fixation and processing variables on immunohistochemistry assay effectiveness, as well as analytic and clinical outcomes, remains largely unknown. In conjunction with the Biospecimen Research Network (BRN), a program within the National Cancer Institute’s Office of Biorepositories and Biospecimen Research, we assessed the current state of the science to both define and steer current and future BRN research initiatives.

We identified potential sources of preanalytical variability, from the experience of the authors and BRN contributors, and existing literature (Table 1). Surveys of published primary research targeting discrete steps of specimen fixation, processing, and storage were conducted to evaluate their possible effects on quantitative and qualitative immunohistochemical analysis. Preanalytical variables with reported immunohistochemical effects are summarized in Table 2. Variables that were not reported to elicit changes in immunostaining (see Table 3), and those that produced antigen-dependent or conflicting results or were underrepresented in the literature, were also highlighted. Evidence-based thresholds for optimal immunostaining were compared with those cited by published immunohistochemistry guidelines and recommendations (Table 4). Although additional sources of preanalytical variability, such as disparities among tissue type or antigen retrieval techniques, may be capable of influencing immunohistochemistry to unknown extents, the scope of the present review was limited to FFPE processing variables.

PREFIXATION

A delay in specimen fixation at room temperature or 4°C does not significantly alter the extent or intensity of immunostaining when less than 12 hours, according to the published literature. Although nonsignificant reductions...
in progesterone receptor (12%) and estrogen receptor (ER; 3%) Q scores—an additive quantification method incorporating the intensity and proportion of immunopositive staining—were observed after a room temperature prefixation delay of 1 hour and 2 hours, respectively, immunostaining for either receptor was not significantly altered even after a delay of 8 hours at room temperature or 24 hours at 4°C. Effects noted after delays greater than 12 hours appear to be antigen specific; for example, the incidence of immunopositive cells was artificially increased, decreased, or unaffected, depending upon the targeted antigen. Current guidelines for ER and progesterone receptor immunohistochemistry from the American Society of Clinical Oncology/College of American Pathologists recommend limiting prefixation delays to 1 hour or less, while an acceptable prefixation delay was not discussed in the remaining guidelines assessed.

To the best of our knowledge, data on the medium used for prefixation storage, use of pathology ink, or method used to set the dye, and their influence on immunohistochemistry, have not been reported.

**FORMalin FixATION**

**Formula**

The concentration, pH, and presence or absence of buffer in the formalin solution used for specimen preservation affects the quality of immunostaining in FFPE specimens. Among the published reports reviewed, most antigens yielded consistent immunostaining and superior preservation when specimens were preserved in 10% neutral-buffered formalin (NBF) at a solution pH of 5 to 7 compared with unbuffered formalin or NBF at a lower or higher pH. However, the optimal fixative for immunohistochemistry may also depend upon the antigen of interest. The intensity of epidermal growth factor receptor immunostaining was superior in specimens preserved with 4% unbuffered formalin compared with 10% unbuffered formalin or 10% NBF, although the extent of immunostaining did not differ among differentially fixed specimens. The type of buffer used for NBF may also affect the quality of immunostaining; T-lymphocyte surface membrane cluster of differentiation (CD) antigens CD3, CD4, and CD8 displayed superior intensity and reduced background staining when specimens were preserved in 10% formalin buffered in Tris solution compared with phosphate-buffered saline (PBS). While published recommendations and guidelines for immunohistochemistry advise fixation in 10% NBF, phosphate buffered saline is cited as the preferred buffer.

We did not find evidence of published reports investigating the potential influences of fixative age, or commercial versus laboratory fixative preparation, on immunohistochemistry.

**Fixation**

Although published recommendations agree regarding the 1:10 volume ratio of specimen to fixative, equivalent immunostaining results were reported for 5 antigens assessed in specimens preserved with volume ratios ranging from 1:1 to 1:20.

The duration of fixation, that is, the length of time a specimen is immersed in a fixative, has been a topic of great interest in the scientific community owing in part to conflicting results and recommended thresholds. While effects associated with underfixation or overfixation hold clinical relevance and include reductions in both the intensity and extent of immunostaining, as well as graded staining from periphery to core in underfixed specimens, definitive thresholds for stable immunostaining remain undefined. Published immunohistochemistry guidelines assessed in the present review agree that 24 hours in fixative is optimal, although minimum...
Table 2. Variables in Specimen Fixation and Processing With Reported Effects on Immunohistochemistry

<table>
<thead>
<tr>
<th>Preanalytic Variable</th>
<th>Analytic Effect</th>
<th>Antigen Dependent</th>
<th>Conflicting Reports</th>
<th>Source, y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td>Alterations in the extent and intensity of immunostaining, as well as nonspecific background staining</td>
<td>No</td>
<td>Yes</td>
<td>Williams et al, 1997, Cerio and MacDonald, 1986</td>
</tr>
<tr>
<td>Temperature and duration</td>
<td>Alterations in the intensity of immunostaining and nonspecific background staining</td>
<td>No</td>
<td>Yes</td>
<td>Pollard et al, 1987, Williams et al, 1997, Cerio and MacDonald, 1986</td>
</tr>
</tbody>
</table>

and maximum fixation durations vary, with a minimum of 6 hours\(^6\) versus longer than 8 hours\(^8\) and a maximum of 48 hours\(^9,10\) versus 72 hours.\(^8\) A survey of the literature introduces further variability, with several studies citing antigen-specific stability with regard to time in fixative.\(^2,13\)

Even for a common antigen evaluated in a particular tissue, ER in breast specimens for example, data conflict, with a minimum of 1 hour,\(^19\) 6 hours,\(^14,17\) or 24 hours\(^6,11\) in fixative required for uniform and reproducible immunostaining. Although maximum times in fixative also vary among reports, the imminent risk for overfixation is comparatively lower, with reductions in ER immunostaining intensity in breast tissue reported after 4 days,\(^11\) 30 days,\(^17\) or 57 days.\(^15\) For other antigens, underfixation is also a greater concern than overfixation.\(^1,12,18\)

Formalin fixation for 24 hours yielded reliable immunohistochemistry results for several antigens in biopsy or surgically procured FFPE specimens.\(^1,2,6,11,13,16,18,20,21\) In fact, only 1 study reported a modest reduction in immunostaining intensity after 22 hours, compared with specimens fixed for 6 hours,\(^22\) after examination of CD5 immunostaining on tissue specimens procured during autopsy.

Given the temporal constraints of the biochemical fixation process, which is dependent on the equilibrium between formaldehyde and methylene glycol,\(^8,10\) and the desire for quick clinical turnaround of pathology laboratory results, several different methods to reduce fixation time have been explored. One such strategy is accelerating the penetration rate of the fixative by increasing the temperature of fixation\(^\) or prefacing specimen immersion with injection,\(^18\) both of which result in modestly reduced immersion times and immunostaining that is equivalent to traditionally fixed specimens. Other novel fixation methods, such as microwave heating\(^23–26\) and ultrasound acceleration,\(^22,27\) have recently been developed and claim to shorten required fixation from hours to minutes. However, recommendations by the Clinical and Laboratory Standards Institute (CLSI) for immunohistochemistry acknowledge that while microwave irradiation can shorten fixation times, specimens should be (1) 5 mm thick or...
less, (2) immersed in formalin for no less than 4 hours before microwave irradiation, and (3) microwaved for 5 minutes or less in 100 ml of formalin.

Guidelines on ultrasound-accelerated formalin fixation are unavailable for immunohistochemistry, and the technique is classified as “experimental” by the CLSI recommendations for immunohistochemistry.

While secondary fixation is common practice in many laboratories, details concerning the reagents, duration, and conditions of secondary fixation often associated with automated tissue processing are frequently unreported in published literature. To the best of our knowledge, variables associated with secondary fixation have not been evaluated for their influence on immunohistochemistry.

| Table 3. Reported Variables in Specimen Fixation and Processing That Do Not Influence Immunohistochemistry |
|-----------------------------------------------|-------------------------------------------------|-----------------------------------------------|
| **Preanalytic Variable** | **Range Examined** | **Source, y** |
| Fixation delay | Temperature | Room temperature, 4°C | Pollard et al, 1987 |
| | | | Williams et al, 1997 |
| | | | Khoury et al, 2009 |
| | | | Di Tommaso et al, 1999 |
| Fixation | Tissue to fixative ratio | 1:1 to 1:20 | Williams et al, 1997 |
| | Method of fixation | Immersion, injection coupled with immersion, microwave acceleration | De Marzo et al, 2002 |
| | | | Chu et al, 2005 |
| | | | Azumi et al, 1990 |
| | | | Hopwood et al, 1984 |
| | | | Login and Dvorak, 1988 |
| | | | Morales et al, 2004 |
| | | | Chu et al, 2006 |
| Postfixation wash | Duration | 6–24 h | Pollard et al, 1987 |
| Tissue processing | Type of automated processor | Carousel, enclosed vacuum | Pollard et al, 1987 |
| | No. and position of specimens | Not specified | Williams et al, 1997 |
| | Duration of clearing | Unspecified reduced and extended duration compared to 4-h control | Williams et al, 1997 |
| | Type of paraffin | Polymer, nonpolymer, microcrystalline | Williams et al, 1997 |
| Section/slide adhesion | Chemical adhesive | Gelatin, APES, APES with glutaraldehyde, poly-l-lysine | van den Broek and van de Vijver, 2000 |
| Storage | Duration of block storage | 2–25 y | Scharl et al, 1990 |
| | | | Bromley et al, 1994 |
| | | | Manne et al, 1997 |
| | | | Shin et al, 1997 |
| | | | Bromley et al, 1994 |
| | | | DI Vito et al, 2004 |
| | | | Jacobs et al, 1996 |
| | | | Wester et al, 2000 |

Abbreviations: APES, aminopropyl-ethoxysilane; PBS, phosphate-buffered saline.

- Antigen-specific effects have been reported.
istry, although CLSI recommends that secondary fixation associated with automated tissue processing be included in the reported fixation duration.10

Fixation parameters that have not yet been evaluated for confounding effects on immunohistochemistry include the size and manipulation of the specimen before immersion (such as sectioning or biopsy procurement ex vivo), the primary method of containment during fixation (eg, a biopsy bag or cassette), and other fixation conditions, such as movement of the specimen within the fixative solution, exposure to light, and the number and relative position of cofixed specimens.

TISSUE PROCESSING

Washing and Short-Term Alcohol Storage

While removal of excess fixative from specimens before dehydration and subsequent processing is not uncommon, little evidence is available concerning downstream analytic effects of such treatment. A single article1 examined the duration of such a wash and concluded that washing fixed specimens in running tap water for 6 hours before dehydration did not alter immunostaining of surface membrane antigens, although a modest reduction in staining intensity was observed after a 24-hour wash.

Although a widely accepted practice and a suggested alternative to prolonged storage in formalin,10 to our knowledge, the potential impacts of alcohol storage on specimen immunoreactivity have not been evaluated in a published report. It is our belief that potential effects associated with short- or long-term storage of specimens in another class of fixative warrant further attention.

Type of Processor

The type of automated processor (carousel versus enclosed vacuum),1,2 and the number and position of specimens within the carrier2 during dehydration, clearing, and paraffin impregnation are reported to not alter the intensity of immunostaining.

Dehydration and Clearing

Although evidence is limited, the type of reagents used for dehydration and clearing may influence the extent and intensity of immunostaining. A single study3 cites that both the extent and intensity of immunostaining were superior in specimens that underwent dehydration in isopropanol compared with ethanol, methanol, acetone, or 8 other reagents. Reported effects attributable to the type of clearing agent used range in severity from no observable difference5 to altered staining intensity28 to alterations in both the intensity and prevalence of immunostaining.1 While both studies investigating this variable cited favorable immunostaining after clearing with chloroform,1,28 the success of other clearing agents was less clear. For example, xylene was reported to be inferior to chloroform and Inhibisol (Bestobell Chemical Products Ltd, Mitcham, Surrey, UK)29; equivalent to chloroform,1 Clearene (Surgipath, St Neots, UK), and an unspecified xylene substitute30; and superior to Histo-Clear (National Diagnostics, Atlanta, Georgia), toluene, and carbon tetrachloride.

The temperature of dehydration and clearing influences the quality of immunostaining, although favorable effects have been reported for temperatures both lower and higher than ambient. Compared with controls that were dehydrated at room temperature, specimens dehydrated in isopropanol at 4°C displayed an increase in the extent and intensity of immunostaining, although temperature-mediated effects were also dependent upon the reagent used.1 Dehydration and clearing of specimens at 4°C attenuated background staining,29 while a temperature of 45°C increased the intensity of immunostaining2 as compared with controls processed at room temperature. Although the total duration of dehydration improved the quality of immunostaining, with 10 hours resulting in more intense immunostaining and reduced background, compared with shorter or longer durations,1,2 the duration of clearing did not appear to influence immunohistochemistry, although data were not shown.2

Details on specimen processing among published recommendations and guidelines are scarce, suggesting only that specimens undergo 1.25 to 15 hours of dehydration, 15 minutes to 3 hours per alcohol stage with 5 recommended stages, with duration dependent upon the type and size of the specimen and the efficiency of the processor.10

Paraffin Impregnation

While the type of paraffin wax (polymer, nonpolymer, and microcrystalline) used for paraffin impregnation does not affect immunostaining,2 the melting point of the polymer paraffin may elicit alterations, as both the extent and intensity of immunostaining were enhanced among specimens embedded in paraffin with a low (45°C) as opposed to a high (65°C) melting point.1 The duration of paraffin impregnation can also impact immunostaining, although reports conflict as to whether shorter (1–2 hours)29 or longer durations (8 hours)2 produce favorable results.

Conversely, recent guidelines and recommendations cite that paraffin with a melting point of 55°C to 58°C and durations of 0.5 to 4.5 hours, 15 minutes to 3 hours per stage with 2 to 3 stages recommended, is preferred for immunohistochemistry.10

Novel Techniques

While recent reports suggest that the time required for sufficient tissue processing may be reduced when performed in conjunction with microwave irradiation26,30 or ultrasound sonication,27 formal published recommendations are not yet available.

Unaddressed Variables

Several factors associated with specimen processing through dehydration, clearing, and paraffin have yet to be examined in detail concerning their impact on immunohistochemistry. These variables include the age of the reagents, which is also related to whether serial stage reagents are freshly replaced or transitioned owing to presumed evaporation or dilution; the number of stages used and the frequency of cleaning and servicing of the automated processor; and the temperature at which specimen positioning within a block occurs.

PARAFFIN SECTIONING

Variables related to sectioning of FFPE specimens, such as the type of blade and frequency of replacement, the frequency of micromere cleaning and servicing, the temperature of the block during sectioning, slide pretreatment, and water bath conditions, if applicable, have received little attention in the literature; however, techniques used to promote paraffin section-slide adhesion have been investigated. While chemicals used to promote section adhesion to
glass slides do not alter immunostaining or antigen stability,
the temperature and duration of slide drying can elicit
significant effects. Published guidelines for immunohisto-
chemistry recommend drying slides at room temperature for
24 hours or 50 °C to 60 °C for a minimum of 1 hour.
While stable immunostaining was reported among slides dried at
room temperature for 24 hours or at 37 °C overnight,
 drying at 60 °C for as little as 4 hours reduced immunostaining
intensity in 23% of target antigens compared with 37 °C
overnight controls, and effects escalated with temperature and
duration, as 69% of target antigens displayed reduced
staining intensity or increased nonspecific staining after an 8-
hour incubation at 70 °C. A different study reported
anecdotal reductions in staining intensity among sections
dried at 80 °C, but not 58 °C or 68 °C, for 16 hours.

**STORAGE**

**Paraffin Blocks**

When compared with freshly embedded controls, para-
fain blocks archived at room temperature for as little as
2 years or as long as 25 years exhibited stable im-
munostaining for most antigens evaluated. In fact, of 6
antigens investigated for storage stability, proliferating
cell nuclear antigen alone displayed storage duration-
dependent reductions in both immunostaining extent and
intensity, effects that were attenuated by antigen retriev-
al. Published immunohistochemistry guidelines concur
with current literature, noting that paraffin blocks may be
stored indefinitely in a “cool place.”

**Paraffin Sections**

Storage of slide-mounted tissue sections can alter
immunoreactivity, although both the time line and
magnitude of effect are antigen dependent. Immunostaining
effects that have been attributed to slide storage
include an artificial reduction2,31,33,35–40 or enhancement45
of staining intensity as well as reductions in the number of
immunopositive cells.2,31,33,35–42 Reports conflict as to whether
these effects translate to alterations in case status.35,41

Although published guidelines recommend storage of
slide-mounted tissue sections be limited to 7 days or
fewer,16 time lines associated with stable immunostaining
appear to be antigen specific.2,31,33,35–44 with immunostaining
altered after 6 days78 or stable for up to 300 days of room-
temperature storage.31 However, thresholds for stable
immunoreactivity of paraffin sections vary widely among
published reports, even for a common antigen evaluated in
a particular tissue. For example, ER immunostaining of
slide-mounted sections was adversely affected after, but
not before, 6 days, 8 weeks,31 12 weeks,37,39 or 6 months31
of room-temperature storage, although 1 report7 cites
stable immunostaining for up to 6 months of storage.
Storage-induced alterations to ER immunostaining include
reductions in staining intensity,73 the number of immunopositive
cells,33,37,40–42 and possibly case status.36,41

While several techniques have been evaluated for their
potential to preserve antigenicity of slide-mounted sec-
tions, a universal preservation protocol has yet to be
identified. Although preservation of antigenicity was supe-
rior among slides stored at −20 °C compared with storage
at 4 °C or 20 °C, slide/tissue section separation frequently
occurred during immunohistochemistry after storage at
−20 °C.40 The efficacy of antigen preservation at 4 °C varies
among reports, with slides stored at 4 °C displaying either
superior31,39 or equivalent immunostaining2,31 compared
with 20 °C controls. Such conflicts regarding the success of
cold slide storage may be influenced by the antigen
targeted or the antibody used, given the differential
stability of 32 antigens evaluated with slides stored at
4 °C, 21 °C, and 37 °C.31 While coating slides in paraffin
before room-temperature storage failed to attenuate stor-
age-induced alterations in immunoreactivity,39,40 paraffin
coating coupled with storage in a nitrogen desiccator
improved the preservation of antigenicity when compared
with untreated slides or either technique alone.38 Although
storage of deparaffinized slides in 10% sucrose in phosphate-
buffered saline at 4 °C prevented the spatially graded
reduction in immunostaining intensity observed among
untreated slides stored at room temperature for 4 weeks,
the duration of stability was not extended beyond 4 weeks.33

**CONCLUSIONS**

Implementation of immunohistochemistry for the analy-
asis of FFPE specimens is both historic and extensive among
research and clinical laboratories. However, given the lack of
alignment among specimen fixation and processing
protocols at different institutions, a central question arises:
Could fixation and processing-related artifacts skew immu-
nohistochemistry data and resultant conclusions? The
results of our literature survey suggest that the answer is
yes. Documented effects of preanalytical variables range in
certainty from the presence of nonspecific background
staining to alterations in immunostaining prevalence,
intensity, and possibly case status. Given the diversity of
effects associated with variations in preanalytical handling,
it is logical to consult available guidelines and recommen-
dations; however, the guidelines we used for comparison in
the present review were not comprehensive, addressing
only 26% of the preanalytical variables we have identified
(Table 1). Further, for several parameters the recommended
ranges were inclusive of values with literature-reported
effects, such as durations of dehydration and paraffin
impregnation. Notably, conflicting data were reported for
several parameters in the literature, even for a common
antigen. Variation among reported effects may be attribut-
able to (1) the tissue type used, as differences in
procurement strategy, specimen density, and cell type
can translate to differences in susceptibility; (2) the
utilization of different antigen retrieval methods, which
may introduce additional or mitigate existing effects
associated with other preanalytical variables; (3) antibody
specificity; (4) differences among the method of quantifica-
tion; (5) the type and strength of the applied statistical test;
and (6) incomplete methodology. Of the 61 preanalytical
variables identified as discrete steps in specimen fixation
and processing (Table 1), only 44% had published results,
with the remaining 56% overlooked or unreported by the
scientific community as a whole. Results of our review
highlight the importance of standardized specimen fixation
and processing, and the continued need for comprehensive
guidelines and rigorous biospecimen research.

The Biospecimen Research Network, a program within
the National Cancer Institute’s Office of Biorepositories
and Biospecimen Research, was developed to address
challenges such as these. Extramural research supported
by the BRN will elucidate effects of preanalytical variables,
as well as provide a solid foundation for evidence-based
best practices for specimen procurement, handling, and
storage. Detailed summaries of existing research on

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preanalytical variables, including articles cited in this review, can be found within the Biospecimen Research Database (http://biospecimens.cancer.gov/brd/; accessed December 2, 2010), a BRN initiative and a free searchable online resource for the location of published peer-reviewed research and review articles.

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References