‘Routine’ tissue preparation in modern diagnostic histopathology.

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Histopathological techniques
The adoption of routine fixation and paraffin wax embedding.

- 1743 Baker - Alcohol preservation of Hydra
- 1851 Clarke - Alcohol/acetic as fixative
- 1879 Fredericq - Alcohol/turpentine/wax (evaporated)
- 1881 Giesbrecht - Alcohol/turpentine/molten wax
- 1893 Blum - Formaldehyde as fixative
- 1899 Hardy - Studied variety of fixative/wax processes
- 1905 Brasil - Formaldehyde/alcohol/acetic fixative
- 1880 – 1910 - Medical schools include microtechnique
- 1910 - 1950 - FFPE becomes prevalent technique

* Bracegirdle 1987

Fixation
Fixation is the single MOST important preparative histological technique.

Poor fixation CANNOT be remedied at any later stage.

The influence of processing
Dependent on quality of fixation
Processing of tissues for histological analysis

Dehydration
Intermediate solvent (Clearing)
Infiltration with support media

Dehydration effects
Following optimal fixation in NBF, ethanol removes some lipids and a few proteins not immobilized by cross-linking. This can produce a small amount of tissue dependent shrinkage (2-15%). Some hardening also occurs. *Ethanol fixation produces tissue dependent shrinkage of 35 - 40% and much more hardening!!!*

Intermediate solvent effects
Xylene is a 'true' clearing agent i.e. it raises the R.I. of tissue.

It also removes some lipids, causes some shrinkage and also some hardening.

Paraffin wax effects
Removes lipids and causes some hardening.

The heated wax causes the majority of tissue shrinkage (may total 30-40%).

May be reduced by minimizing the heat shock on transfer from xylene to molten wax.

*(Time may be shortened by agitation and negative pressure)*
### Effects of fixation/processing

- Loss of constituents
- Shrinkage
- Hardening
- Change in optical properties
- Inactivation of most enzymes
- Change in acidophilic/basophilic properties
- Destruction or masking of antigen epitopes
- Change in morphology

**ALL OF THESE EFFECTS ARE MINIMIZED FOLLOWING OPTIMAL FORMALDEHYDE FIXATION!!**

### The nature of fixatives

<table>
<thead>
<tr>
<th>Non-Coagulant fixatives</th>
<th>Coagulant Fixatives</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Formaldehyde</strong></td>
<td><strong>Alcohol</strong></td>
</tr>
<tr>
<td>Protein secondary structure intact. Only modifies tertiary and quaternary structures, (Methylene bridge cross-links) mostly (90%) retrievable, with little loss (&lt;1%) of protein.</td>
<td>Protein primary structure intact. Alters secondary and tertiary structures, (Hydrophilic/phobic inversion) often irretrievably, with loss of up to 40% of protein.</td>
</tr>
</tbody>
</table>
**Formaldehyde fixation**

- Non-coagulant fixative
- Fast penetration
- Slow fixation
- Little loss of constituents (< 1%)
- Little shrinkage
- ‘Soft’ fixative
- Many effects are reversible
- Most realistic overall morphology, allows widest range of histochemistry

**Alcohol fixation**

- Coagulant fixative
- Medium penetration (K = 1.0)
- Fast fixation (fixes as it penetrates)
- Loss of constituents (↑40%)
- Causes shrinkage
- ‘Hard’ fixative
- Not readily reversible

Great for nuclear morphology and staining of nucleoproteins, restricts range of histochemistry

**Fixation Reality #1**

Formaldehyde fixation provides the most realistic overall morphology and becomes the standard fixative for the majority of routine diagnostic histopathologists!
Formaldehyde allows the widest range of histochemical stains.

‘Routine’ tissue preparation

- Development of tissue preparative techniques
- The current state of the art
- Fixation re-visited
- Effects on staining
- Effects on QA
- What we need to do to improve

1950’s processor (optional 24 hour and 7 day timers)

Formaldehyde fixation – automated processing
Fixation

Reality #2

Following formaldehyde fixation; automated processing techniques provide an advantage in speed and ease of use, with no loss of morphology!

Fixation

Reality #3

Formaldehyde fixation becomes integrated with automated processing, providing a further advantage in speed and ease of use.

The change in morphology is deemed acceptable and becomes ‘Routine’!
Technical Quality Standards

Few universal standards are applied to routine histological techniques.

Standards that exist are usually of a local, subjective nature, such as:

‘our pathologist likes it this way’
‘we’ve always done it this way’
‘it looks alright to me’

Variations in one histological technique are often introduced as a local response to a real or perceived problem.

The root cause of the ‘problem’ may actually lie in another histological technique.

These variations are empirically derived and are spread anecdotally.

This causes a wide range of reported results.

Small GI biopsy ‘Routinely’ NBF-fixed and processed overnight.
Feels ‘Gritty’ on sectioning

THE ASSERTION:
The tissue is;
‘over-fixed’
‘over-dehydrated’
‘over-processed’

PROPOSED SOLUTION:
Change the pertinent processing times!
THE REALITY:
The section was cut too rapidly, resulting in Knife edge vibrations or ‘chattering’.

THE SOLUTION:
Cut the section slowly.
The ‘chatters’ disappear!

The same small GI biopsy ‘Routinely’ NBF-fixed and processed overnight. Sectioning performed at a slower rate, no longer feels ‘gritty’

‘Routine’ tissue preparation
Development of tissue preparative techniques
The current state of the art
Fixation re-visited
Effects on staining
Effects on QA
What we need to do to improve

Fixation myths
Formaldehyde fixes at a rate of 1.0 mm/hour.
**Formaldehyde fixation**

**How long will it take to fix?**

Tissue slice = 5 mm thick  
Core biopsy = 1.5 mm thick

5 hours?  
1.5 hours?

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**Formaldehyde fixation**

What is the fixative penetration rate?

Medawar, (1941) established that fixatives obey the diffusion laws. That is, the depth penetrated is proportional to the square root of time. Each fixative has a unique coefficient of diffusibility, designated K. Penetration rate may be determined from the formula:

\[ d = K \times \sqrt{t} \]

Where \( d \) = depth in mm  
\( K \) = the Medawar coefficient  
\( \sqrt{t} \) = the square root of fixation time in hours.

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**Formaldehyde fixation**

What is the coefficient of diffusibility (K)?

Tellyesnicszky (1926) used thick tissue and long times. \( K = 0.78 \)

Medawar (1941) used Plasma clots. \( K = 5.5 \)

Baker (1958) used Gelatin/Albumen models. \( K = 3.6 \)

(Helander’s (1994) data indicates K must be AT LEAST 2.0 and probably closer to 3.5)

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**Formaldehyde fixation**

Penetration rate

Time = 1 hour  
\( K = 3.6 \) (Baker)  
Penetration = 3.6 mm

Size = 5 mm
Fully penetrated
**Formaldehyde fixation**

How long will it take to fix?
Penetration time at $K = 3.6$

<table>
<thead>
<tr>
<th>Time</th>
<th>Depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
<td>3.6 mm</td>
</tr>
<tr>
<td>4 hours</td>
<td>7.2 mm (1.8 mm/hr)</td>
</tr>
<tr>
<td>16 hours</td>
<td>14.4 mm (0.9 mm/hr)</td>
</tr>
<tr>
<td>64 hours</td>
<td>28.8 mm (0.45 mm/hr)</td>
</tr>
<tr>
<td>256 hours</td>
<td>57.6 mm (0.225 mm/hr)</td>
</tr>
</tbody>
</table>

*(to double the depth takes 4x the time)*

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**Fixation myths**

Formaldehyde fixes at a rate of 1.0 mm/hour.

Small pieces of tissue fix faster than larger pieces.

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**Formaldehyde fixation**

How long will it take to fix?

Penetration time at $K = 3.6$

<table>
<thead>
<tr>
<th>Time</th>
<th>Depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 minutes</td>
<td>&lt;5 minutes</td>
</tr>
<tr>
<td>10 hours</td>
<td>1 hour</td>
</tr>
<tr>
<td>1.0 cm thick tissue slice requires ~36 hours</td>
<td></td>
</tr>
</tbody>
</table>

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**Formaldehyde fixation**

How long will it take to fix?

The formaldehyde paradox.

Histologists have known for more than 70 years, that fixation of tissue in formaldehyde demonstrates a bizarre effect.

Namely, that formaldehyde is one of the fastest fixing agents to penetrate tissue but one of the slowest to fix.

The paradox was explained (Burnett) in 1982. However, many histologists remain unaware of the implications.
**Formaldehyde fixation**

How long will it take to fix?

The chemical reaction.

1) Formaldehyde covalently binds to reactive side chains on proteins at random to form unstable addition complexes.
2) Once a sufficient number of addition complexes are formed, they may slowly cross-link to each other by formation of methylene bridges.
3) Progressive formation of cross-links promotes gel formation and confers stability on the tissue.
4) These reactions are readily reversible.

**The reaction rate (The ‘Clock’ reaction).**

1) In aqueous solution, formaldehyde is hydrated and mainly exists as methylene glycol.
   (< 1 part in 100,000 exists as ‘free formaldehyde’)
2) Methylene glycol penetrates the tissue rapidly but does not fix.
3) Binding of the little available ‘free’ aldehyde starts the clock reaction and allows slow decomposition of glycol to aldehyde over several hours.

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**14 C labeled formalin binding time**

- Fox, et al 1985
- Helander, 1994

- 16 μm section
- 24 hours

4 x 4 x 4 mm tissue cube = 25 hours
Formaldehyde fixation

For initial stabilization of fixation to occur, binding time is crucial, NOT penetration time.

= 24 hours minimum for a 1.5 mm thick core biopsy.

= 24 hours minimum for a 5 mm thick tissue slice.

Binding time + penetration time = Reaction rate!

Fixation myths

Formaldehyde fixes at a rate of 1.0 mm/hour.

Small pieces of tissue fix faster than larger pieces.

The optimal fixation time for formaldehyde is 24 hours.
Fixation

Reality #4

Acceptable morphology on routine H&E stain does NOT correlate with acceptable staining by other methods!

Especially with Immunohistochemistry!

Why would anyone use Immunohistochemistry?

IHC provides a final visual label on microscopic entities of interest, many of which cannot be identified by other histochemical techniques.

IHC can answer the questions;

- What is it?
- Where is it?
- How much is present?

Immunohistochemistry

Many different protocols in use.

Sensitivity and Specificity varies with small changes in protocol.

Wide range of reported results.

Immunohistochemistry

Can provide answers to additional questions, such as;

- \textit{(Prognosis)}
  What is the likely future course of the patient’s disease?

- \textit{(Predictive)}
  How will the disease respond to therapy?
**Immunohistochemistry**

The current and future use of IHC as a standalone diagnostic, prognostic and predictive tool, demands reliable and reproducible results.

These results may determine patient treatment!

**Immunohistochemistry**

Current estimates indicate that IHC is required in up to 25% of malignancies. This will increase as proteomic studies produce more targeted therapeutic agents.

Standardized IHC kits for antigens of interest and automated IHC instruments help, but variability of results is still problematic.

**Quality in histotechnology**

Many histopathology laboratories use both ‘quality control’ (QC) and ‘quality assessment’ (QA) activities for the various individual steps involved in completing the daily workload.

Unfortunately, little attention is given to the overall integration of these daily activities, to assess ‘total quality’ (TQ).

**Quality control (QC)**

QC activities are prospective, *i.e.* they look forward at what will happen if all the steps in the process are followed.

QC defines a product’s quality and imparts to it the credibility needed for its intended purpose.

QC activities are the result of advanced planning and are applied to everything that contributes to the final product (on-line controls).
Quality assessment (QA)

QA activities are retrospective, i.e. they look back at what has happened, with a view to measuring the degree to which the desired outcomes are successful.

QA provides opportunities to subsequently modify the processes contributing to the final product (off-line controls).

Total quality (TQ)

TQ, or the ‘total test’ approach is a more holistic look at all the steps, in all the various processes used, from obtaining the sample until the final reporting of results.

TQ involves integrating all of the QC and QA findings and understanding how changes to any of the various processes will affect the final outcome.

The latter may involve experimentation to provide provenance for any proposed modification of the processes and the consequent QC and QA activity.

Immunohistochemistry

IHC is technically complex.

No aspect of this complexity can be ignored.

QC of the IHC procedure alone is insufficient.

QA and particularly EQA helps to provide valuable additional information for TQ.
PATH-0402 Overall Vimentin scores

Immunohistochemistry
The Total Quality Approach

Looks at global factors that may influence IHC performance, from the collection of the sample to final interpretation.

Both off-line and on-line controls are utilized to give total test performance.

The Total Quality Approach

Standardize factors that can be standardized.

Understand the effect of those factors that cannot be standardized.

Optimize the IHC procedure to accommodate all factors.

Validate the results!

Immunohistochemistry

Immunohistochemical techniques rely upon the steric interaction (best fit) between the antibody paratope and the matching epitope of its target antigen.

Recognition critically depends upon the epitope remaining unaltered and available to react.
Factors effecting the IHC Detection threshold

- Biological variation
- Sample collection
- Fixation/processing
- Section thickness
- Section pre-treatment
- Antibody-type/clone
- Antibody-dilution
- Sensitivity of detection reagents
- Histochemical reaction
The impact of the nature & duration of fixation and processing, on IHC detection threshold is

**ENORMOUS!**

(90% of IHC staining problems)

The routine histological section:

Is it really formaldehyde fixed?

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Reality #5

No universal standard exists so what, exactly, IS a routinely formaldehyde-fixed, paraffin section?

Your routine or mine?

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*Routine* A & B
Reality #6

Routine fixation/processing consists of allowing tissues to fix for variable periods of time.

The actual fixation time being dictated by the start time of the processing machine!

Formaldehyde fixation

Forms methylene bridges at reactive side chains
Fast penetration (3.6 mm in 1 hr)
Slow fixation (24–72+ hrs)
many effects reversible (<24 hrs)
No shrinkage
“Soft” fixative
Mildly cross-links proteins
Most versatile

14C labeled formalin Binding (24 hr)
Reversal time at 25°C

The nature of fixatives

Coagulant Fixatives

Alcohol
*during processing

Non-Coagulant fixatives

Formaldehyde

Protein secondary structure intact. Only modifies tertiary and Quaternary structures, (Methylene bridge cross-links) mostly (90%) retrievable, with little loss (<1%) of protein.

Coagulant Fixatives

Protein primary structure intact but alters secondary and tertiary structures, (Hydrophilic/phobic inversion) often irretrievably, with loss of up to 40% of protein.
The routine histological section reality #7:
Most are NOT really formaldehyde fixed!
They are variably fixed by a combination of formaldehyde and alcohol.

Routine formaldehyde fixation/processing;
Results in;
• variable morphology and cell content
• variable shrinkage and hardening
• variable masking/destruction of epitopes
• variable porosity
• variable basophilic/acidophilic relations
• variable intensity of stains
variable success/failure of staining techniques!
Impact of ‘routine’ tissue preparation on HER2 IHC positivity rates (2002).

‘In-house’ positivity rate = 22% (+ 5% equivocal)  
(ALL fixed for minimum of 24 hours, including core biopsies)

‘Referred-in’ positivity rate = 8% (+ 20% equivocal)  
(‘routinely’ fixed)

‘Over-all’ positivity rate = 13%  
(Accepted range = 18 – 22%)
Myths

‘Formalin fixation for more than 24 hours is ‘Overfixation’ and will destroy immunoreactivity’.

Reality #9

There is NO such thing as ‘Overfixation’ in formalin. Progressive cross-linking does occur over time. This may lead to masking of antigens. This does not occur within any reasonable time frame (5-7 days) for the majority of antigens of clinical interest.

We have tested ER, HER2 and CD117 for up to 90 days of fixation time, finding NO significant loss in immunoreactivity after 24 hours.

Fixation

Fixation is the single MOST important preparative histological technique.

Poor fixation CANNOT be remedied at any later stage.
‘Routine’ tissue preparation

Development of tissue preparative techniques

The current state of the art

Fixation re-visited

Effects on staining

Effects on QA

What we need to do to improve

Reality #10

Standardize fixation!

Standardize fixation times for all tissues requiring prognostic/predictive markers!

(24 hour minimum in NBF)

Is this the time to duck?

Strategies

Standardize fixation by:

1) Cut thin (3-4mm) blocks.
2) Use a 20:1 fixative – tissue ratio.
3) Adopt a routine minimum 24hr fixation time.

OR

4) Fix only ‘Special tissues/blocks’ for 24hrs.

OR

5) Stay with variable ‘routine’ fixation and HOPE!

Consequences

1) Improved fixation and processing.
2) Improved maintenance of pH and fixation.
3) Extension of current turn-around time (TAT).

OR

4) Only ‘Special tissues/blocks’ TAT affected.

OR

5) No standardization!

Standardized fixation improves IHC performance!
Recommended fixation times

Estrogen Receptor protein.
OAP/CAP- SenGupta et. al.

Fixation in buffered formaldehyde for 12 - 24 hours.
AJCP, 120(1) July 2003. Goldstein et. al.

“Minimum formalin fixation time for reliable IHC ER results is 6 - 8 hours regardless of the type or size of specimen”.

Recommended fixation times

HER2 protein.
HercepTest™.

“Tissues from the biopsy should be blocked into a thickness of 3 or 4 mm and fixed for 18 - 24 hours in neutral buffered formalin (NBF)”.

CAP/ASCO. “Fixation in NBF for 6 - 48 hours”.

QMP-LS.
“Fixation in phosphate buffered formaldehyde for a minimum of 24 - 48 hours”.

Breast Cancer. ER = Positive (range of expression)
Standardized IHC protocol
**Myths**

'The IHC control tissues should be fixed and processed in a similar manner to the test sample'.

*CLSI guidelines for IHC*

**Reality #11**

Yes they should, but unless you have adopted a standard fixation time for ALL test samples, this is virtually impossible!

In practice, a strategic fixation time set on a given control tissue will satisfy this requirement.
Myths

‘Every antibody should have ‘AR’ pre-treatment’.

Reality #15

Each antigen-antibody interaction is unique. The necessity for any form of pre-treatment is dictated by the response to fixation of individual antigen epitopes.

Pre-treatment

May un-mask or improve the accessibility of epitopes.
May damage other epitopes.
May enhance unwanted cross-reactions.
May degrade morphology.

All are dependent on fixation/processing!

Pre-treatment

There are NO standards for pre-treatment!

For proteolytic agents, standardize the time, temperature and concentration for use.
(activity rating in units/mg solid)

For HIER, standardize the time at temperature, the buffer concentration and pH.

These can ONLY be truly standardized if the fixation is!
Use The Total Quality Approach

Standardize factors that can be standardized.

Understand the effect of those factors that cannot be standardized.

Optimize the IHC procedure to accommodate all factors.

Validate the results!

“Any smoothly functioning technology has the appearance of magic”

Arthur C. Clark