Controls in Histopathology: What can and what should be controlled

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What should be controlled?

- Everything!
  - Procedures
  - Personnel
  - Equipment
  - Records
  - Especially pathologists and residents!
The GLP Concept

GLP means Good Laboratory Practice

- Many definitions in Federal documents
- Common sense rule
  - All activities documented in SOP documents
  - Each SOP must have control or verification associated

GLP continued

- Analyze each step of every procedure
  - Goal is to insure repeatability
    - From day to day
    - From specimen to specimen
  - Even the most straightforward daily tasks may cause problems
  - Above all, SOP’s assist in avoiding mistakes, *if followed*
Standard Procedures

- Provide a way to solve problems
- Insure consistency and quality
  - Everyone performs task same way
  - Sets laboratory standard, not individual
- Based on specimen quality, not individual

SOP’s must be dynamic

- Procedures should be reviewed annually
- When process fails
  - Should address failure analysis
  - Provide framework for resolution
  - Investigation and resolution must be documented
  - Provide mechanism for revision of SOP
A brief history of histology control procedures

- Early texts still cited
  - Technology is much different today
  - Process controls are much better understood
  - Do you really need a procedure to fold paper boats?
  - How about those temperature logs?

Failure modes should be considered

- Temperature systems monitoring is probably a waste of time
  - Heating and cooling devices fail catastrophically
  - No evidence that temperature monitoring predicts eminent failure
Workflow determines needed procedures

Any procedure that can produce variation in the daily workproduct (stained slides) must have established control procedures.

Control procedures must be validated!

Specimen controls

- Identifiers
  - Carried along with specimen
- Time
  - Collection time
  - Grossing time
  - Total fixation time
Fixation

- CAP guidelines
- Technical guidelines
  - How does fixative work
  - Is it being used appropriately
  - How do you verify proper use
  - Different fixative require different procedures

Impact of Fixative Additives

- Formalin is generally buffered
  - Commonly with phosphate buffers
    - Traditional
    - Cheap
  - Buffers supposedly eliminate “formalin pigment”
    - Not a significant problem for most specimens
Phosphate buffers

- Have very low solubility in ethanol of high concentration
  - Any alcohol concentration over 75% will cause phosphate salt precipitation
    - Results in “gritty blocks”
    - May plug up plumbing of tissue processors
    - Must be washed out of specimen before dehydration

Why do we dehydrate

- Paraffin is not miscible with water or with ethanol
  - A transition solvent is required
    - Transition solvent must be miscible with both ethanol and with paraffin
  - Many possible transition solvents
    - Each has unique properties, and may require different control procedures
The mushy block problem

- Often block center will not contain paraffin
  - Invariably caused by incomplete dehydration
    - Specimen too large
    - Dehydration steps too short
    - Poor quality dehydrant

Transition solvents

- Classic example is xylene
  - Quite toxic, so many labs try to avoid
- Aliphatics are common replacement
  - Much lower tolerance for water
  - Many have lower paraffin solubility
- Isopropyl alcohol is directly miscible with paraffin
Quality of fixative solutions

- Difficult to monitor using commonly available histology laboratory facilities
  - Final block quality is alternative
  - Should implement incoming solution QC
  - Different procedures for each type of fixative

Embedding

- Needs procedures
  - Types of paraffin
    - Incoming QC
    - Melting behavior
    - Impurities
  - Specimen
    - Size
    - orientation
Sectioning

- Microtome maintenance
- Blades
- Section thickness
  - Not necessarily same as microtome setting
  - Not easy to measure
  - Practical thickness evaluation

Section Traceability

- Slide must be traceable to individual cutting sections
  - Without this, can’t correct issues
  - Important for continuing education of staff
- Uniform quality requires same level of competence for all
Slide drying

- Highly variable, laboratory to laboratory
  - Avoid temperatures above melt temperature of paraffin
  - Extended drying times may induce polymerization of polymers
  - Microwaves are always suspect

Slide drying continued

- Purpose of drying is to remove water
  - Depends on slide surface
  - Depends on type of slide adhesive
  - Time to dryness should be monitored
    - Exposure to high heat while tissue if dehydrated should be avoided
Deparaffinization

Most laboratories don’t adequately remove all paraffin
- 2005 paper found paraffin residue in most stained sections
- Common protocols are too short
- Aliphatics require longer exposure times
- Patch staining may result

Deparaffinization

Classic texts recommend three changes, five minutes each, in xylene
- Those recommendations were for pure paraffin or paraffin with beeswax
- Modern paraffin contains polymers that may be more difficult to remove
Staining control

- The H & E stain is the “gold standard”
  - But it isn’t controlled at all!
  - Various formulations of stain available from manufacturers
  - Labs use different protocols, from lab to lab
  - Individual labs vary color balance depending on pathologist

What is a good stain?
Subjective Evaluation

- Eye as a sensor
  - Adaptive in sensitivity
  - Limited density range
  - Exquisite color definition
    - But no two individuals see color same way
    - Impossible to describe color to another individual

Subjectivity is Bane of Pathology

- Pathology is a craft, learned at feet of "master"
- Even experts disagree
- Repeatability of single individual is suspect
- CAP has documented variability with respect to IHC evaluation
How should stains be evaluated?

- Must use instruments – image analysis
- Remove subjectivity
- Image analysis requires controls for stain
  - Who determines control?
  - How should the control behave?
  - Is it possible?

Empirical Stains vs Histochemical Stains

- Empirical stains are used strictly for morphology
  - H & E and most special stains
- Histochemical stains are actually a chemical reaction
  - Controls can be designed for each chemical
The role of water quality

- Most empirical and histochemical stains are dependant on pH
  - Laboratory water pH varies widely
    - Same water supply may have seasonal variation
    - Water may contain variety of contaminants
      - Particulates
      - Microorganisms

Monitoring water

- pH routinely (recommended weekly)
- Check for particulates
- Check for microorganisms
- Institute methods for correction of any issues found
- Stain quality may be early indicator of water issues
Stain shelf life and stability

- Pre-mixed stains subject to environmental conditions
  - Shipping
  - Storage
  - Humidity

Incoming stain QC

- Visual check mandatory
- Recommend pH check
- If possible, dye content analysis
  - Spectrometer
  - Chromatography for multiple dye stains
Biological stains (immunoreagents)

- More susceptible to shipping and storage conditions
  - Heat sensitive
  - Can be degraded by microorganisms
    - The “sniff” test
    - The visual test

Assessing Stain Quality

- CAP guidelines require daily evaluation of stain quality by supervisory pathologist (still subjective)
- Comparison of slides in batch stain run
  - Must account for differing densities of stain
  - Must account for differing color balance
Coverslipping

- Many potential issues
  - Bubbles
    - Air
    - Water
  - Inappropriate amount of medium
    - Underfill
    - Overfill

Think Process Control

- Tasks are complex processes
  - Understand each step of process
  - Document how to perform
  - Report any deviations and document
  - Validate all processes
    - Revalidate when any element of process changes
Subjective Evaluation is Culprit

- Subjectivity limits standardization
  - No baseline for performance
  - No agreed upon standards
  - No repeatability
  - Lack of standardized specimens limits quantifiable evaluation

Subjective vs Quantitative

- Subjective evaluation has difficulty evaluations density levels
  - IHC stains are evaluated into four levels
    - Lack of reproducibility
    - Lack of agreement

- Quantitative assessment even with simple instruments can yield 256 density levels
Quantitative assessment

- Specimen preparations (stained slides) are too variable for measurement
  - Minor differences in fixation change stain density
  - Minor differences in processing change stain density
  - Minor differences in section thickness change stain density

Quantitative assessment

- Without defined, reproducible control objects, quantitative assessment is hopeless
  - With a defined control object, it would be possible to account for many of the differences due to current processes
  - Section thickness of specimens is still a significant issue
Pathologists are not Technologists

- CAP guidelines are produced by pathologists
  - Her-2 guidelines have been problematic
    - Have not resulted in increased accuracy of assessment
  - ER-PR guidelines *may* be an improvement
    - Technical expertise contributed to recommendations

Many misconceptions

- Impossible to make meaningful recommendations if process is not understood
  - Her-2 fixation recommendations
  - Published literature on Image analysis of DAB
Immunostain Controls

- All current recommendations based on primary antibody
  - Positive control
  - Negative control
  - Negative serum control
    - Only control that may provide some indication of secondary stain process

Immunostains

- All current protocols use some version of a “sandwich” assay
  - Multiple reagents used to provide amplification
    - Either two or three amplification steps
- Almost all current protocols utilize polyvalent (multiple species) secondary reagents
Immunostains

- Final color developed by enzyme activity
  - In US, most common enzyme is peroxidase, demonstrated by Diaminobenzidine (DAB)
  - In Europe, most common enzyme is alkaline phosphatase, using a red chromagen

Characteristics of DAB

- DAB is a particulate
  - Particles were used as label for electron microscopy
  - Particles cannot be measured by light absorption
    - DAB scatters light
    - Produces a non-linear signal
      - Cannot equate to stain density
DAB characteristics

- Other DAB chromagens may be measured
  - AEC is a true light absorber
  - Vector NovaRed is also a true absorber
- Brown color of DAB is a complication in double staining protocols

Consequences of Polyvalent Secondary

- Current polyvalent secondary reagents are mixture of anti-mouse and anti-rabbit
  - May have different titer (sensitivity) for each species
  - May have different shelf life characteristic
- Need to run both mouse and rabbit controls for secondary
An Immunostain Process Control

First implementation

100% R, 0% M
100% R, 50% M
100% R, 100% M
0% R, 100% M

100% R, 25% M
100% R, 75% M
75% R, 100% M
25% R, 100% M
Additional Work

Spots and Tissue
Tissue and varying spot size

Spot Consistency