Assessing the Quality of Tissue Processing and the Performance of Peloris™ using the Leica Microsystems Scoring System

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Abstract

This paper outlines the test procedure and scoring system which has been used to objectively evaluate the quality of tissue processing during the development and comparative evaluation of the Peloris™ tissue processor and as a mechanism for optimising standard processing protocols. The source and content of suitable specimen panels are described and the need for standardization of every aspect of specimen handling, processing and section preparation is emphasised. The scoring system assesses 23 parameters in six groups and is based on a three point scale. The system effectively allows comparisons of processing runs to be made using different processors or different protocols on the same processor.

Introduction

Various approaches have been taken in assessing the quality of fixation, processed tissues and tissue sections. Methods have ranged from the purely subjective, where a morphological description is provided[1], simple grading based on descriptive criteria[2], score generation based on a 4 point[3, 4] or 5 point scale[5] or more complicated systems of scoring[6].

In order to thoroughly test Peloris and because of the importance of optimising our recommended processing schedules, we have devised a new, comprehensive test procedure and scoring system. This allows us to objectively evaluate the overall quality of tissue processing, compare the performance of Peloris with that of other processors, and compare processing protocols to refine our recommendations. A general outline of our test procedure and scoring system is provided. We are publishing our procedure in detail because we feel it will be of value to client laboratories that wish to modify processing schedules or devise new ones for their own purposes, or compare the results obtained from different schedules or different processing equipment to those obtained using Peloris.

Method

Basically our procedure involves processing a representative panel of specimens of a standard size, which have been collected and fixed under controlled conditions. Embedding, sectioning, mounting, drying and staining are completed using standardized methodology. As each block is sectioned, it is scored for various cutting and mounting qualities, and after a one week period, it is evaluated for its stability on storage. The sections prepared from each block are scored for their physical quality, the quality of microscopic preservation and staining quality. Multiple blocks of each tissue type are evaluated to allow a statistically significant overall score to be calculated. This score is then compared to that obtained from blocks from a control processing run using a reference protocol (this might be the schedule we are trying to improve on), or a reference processing machine. The specimen panels for test and control runs are obtained from the same source at the same time and, with the exception of the protocol employed and/or the processor used, are treated in an identical fashion in every respect.

We believe it is essential to always compare a test group against a valid control because of the relatively small differences that may exist between the results produced by different protocols (or processors), and the potential for differences in the quality of fixation between tissues taken at different times and from different sources.

It is important to obtain specimens of an appropriate and consistent quality for our panels. Because of the difficulty in getting suitable human tissue on demand, we have very successfully used pig tissue obtained under controlled conditions from an abattoir. For field trials conducted in large hospital laboratories, we have been able to use human tissue. Any laboratory undertaking comparative evaluations of tissue processing will need to think carefully about a reliable source of material for their specimen panels.

Tissue panel composition

Our objective is to ensure that we always have consistent processing parameters so that we can assess the performance of the processor and protocol with confidence.

Assuming the protocol being evaluated is to be used to process a mixture of different specimen types, those included in the panel must contain a comprehensive range of tissue types with a variety of histological characteristics. They should range from delicate cellular tissues such as kidney, to dense fibrous or muscular tissues found in the gastrointestinal tract and heart. They should represent the extremes of the range of specimens encountered in actual laboratory practice.

If human tissue is unavailable for reasons alluded to above then, of the various animals available, the pig provides specimens closely resembling human tissue. A convenient, regular and reliable source of specimens is essential as a number of runs may be required in order to fully refine a new processing schedule. The following is used for an initial panel for preliminary experiments:

Animal	Specimen type	Source of specimes	Rationale for choice
Pig	Kidney	Abattoir	Contains a range of epithelial tissue with characteristic morphological features and delicate basement membranes and stromal tissue. Fine structural detail allows objective assessment of fixation and processing quality
Pig	Liver	Abattoir	Contains hepatocytes with characteristic morphological features in association with delicate sinusoidal vessels. Also present is connective tissue containing vessels and ducts. This tissue is notorious for becoming brittle and cracking during processing
Pig	Skin	Abattoir	Contains keratin, epithelial tissue, a range of connective tissue types and muscle. The layers in skin can separate and keratin can become very brittle if poorly processed
Pig	Small intestine	Abattoir	Gastro-intestinal tract specimens possess a thick layer of glandular epithelium with underlying layers of connective tissue and smooth muscle. It is subject to rapid autolysis prior to fixation and sometimes the layers separate during processing and section preparation
Pig	Spleen	Abattoir	Spleen is representative of the lymphoid and haematopoietic organs containing lymphocytes, elements of circulating blood, a fibrous capsule and delicate reticular and endothelial stroma. Spleen is very haemorrhagic tissue and as such can easily become brittle on processing

Table 1. Peloris processing schedules

Pancreas, some other endocrine glands and some tumor types have a similar composition to kidney and liver. Lymph nodes and bone marrow have features in common with spleen as do some lympho-reticular neoplasms (tumors). Exocrine glands such as salivary gland, some components of the reproductive tracts (vas deferens, fallopian tube) and some fibrous or muscular tumors have histological processing characteristics similar to those of the gastrointestinal tract. Skin contains a range of tissue types including adipose tissue and has features common to breast tissue.

The initial panel of specimen types chosen would provide a clear indication as to the suitability of a processing protocol and the effectiveness of the processor used. It should be noted that for final testing an expanded panel which includes stomach, small intestine, colon, heart, uterus (myometrium) and brain or spinal cord, as well as kidney, liver, skin and spleen is generally used.

Tissue specimen dimensions

We have found the following specimen dimensions to be appropriate:

1. 3mm diameter core biopsy, 10mm long for short protocols
2. 6mm diameter core biopsy, 10mm long for short protocols
3. 3mm x 5mm x 10mm block for longer protocols
4. 6mm x 5mm x 10mm block for longer protocols

To establish the limitations of your processing technique, it is useful to include specimens of a larger or smaller size than what you may consider ideal (or normally acceptable) in your laboratory.

Fixation

We have used two separate procedures for fixation. The first is "normal" fixation which will be used to ensure that the tissue is completely and consistently fixed. This is intended to largely eliminate fixation as a variable affecting the overall quality of processing. The second procedure is to simulate human tissue being delivered directly from the operating theatre for "rapid" processing.

Animal	Rationale for choice
Type	Formalin
Composition	10% buffered formalin
Volume fixative:tissue	50:1
Duration	2-7 days
Time prior to fixation	<30 minutes
Storage temperature	Room temperature
Storage conditions	In sealed container

Table 2a. Fixation parameters for specimen panels: normal

Animal	Rationale for choice
Type	Formalin
Composition	10% Buffered formalin
Volume fixative:tissue	50:1
Duration	2-7 days
Time prior to fixation	<30 minutes
Storage temperature	Room temperature
Storage conditions	In sealed container

Table 2b. Fixation parameters for specimen panels: rapid (straight from abattoir)

Specimen handling

The manner in which specimens are handled at every step prior to processing has the potential to affect the appearance of processed tissue. We have encountered situations where it is difficult to determine whether an artefact visible in the tissue is due to a processing problem or has resulted from something done to the tissue prior to processing.

Parameter	Detail
Rinsing prior to fixation	This is not acceptable (we must ensure that this is not done by abattoir workers prior to receipt)
Sharpness and type of instrument	Fresh dissection instrument for every delivery of tissue
Dissection technique and mechanical distortion	Dissection technique must be standardized as far as possible so that specimens are fully comparable from batch to batch and do not contain crush artefact, ragged margins or other effects due to mechanical distortion
Drying and delays prior to fixation	This will not happen if procedures are followed; these could happen if abattoir workers are not properly briefed on our requirements

Table 3: Tissue handling considerations for specimen panels

Dissection technique

As far as possible we define the technique to be used to prepare specimens for our panels. It is preferable if a single staff member deals with all the specimens for any runs that are to be compared so that a high level of consistency can be achieved.

Parameter	Detail
Kidney	(a) Use a sharp biopsy punch tool to take a 3mm and a 6mm diameter core of tissue close to and parallel to the capsule, to a depth of approximately 4mm (b) Use a sharp scalpel to cut a 3x5x10mm block and a 6x10x20mm block of tissue that includes mainly cortex and some medulla
Liver	(a) Use a sharp biopsy punch tool to take a 3mm and a 6mm diameter core of tissue perpendicular to the capsule, to a depth of approximately 4mm (b) Use a sharp scalpel to cut a 3x5x10mm block and a 6x10x20mm block of tissue representative of the sample provided
Skin	(a) Use a sharp biopsy punch tool to take a 3mm and a 6mm diameter core of tissue perpendicular to the skin surface, to a depth of approximately 4mm (b) Use a sharp scalpel to cut a 3x5x10mm transverse slice and a 6x10x20mm transverse slice of tissue showing all skin layers.
Spleen	(a) Use a sharp biopsy punch tool to take a 3mm and a 6mm diameter core of tissue perpendicular to the capsule, to a depth of approximately 4mm (b) Use a sharp scalpel to cut a 3x5x10mm block and a 6x10x20mm block of tissue which contains trabeculae, and white and red pulp.

Table 3: Tissue handling considerations for specimen panels

Enclosing specimens and loading processor

All specimens are loaded into the same types of cassettes with or without foam inserts as appropriate. The number of cassettes in a processing run are standardized as are the positions of the cassettes in the basket in the processor retort. If we wish to evaluate a schedule simulating a full load of specimens without actually using tissue in each cassette, then we include foam biopsy pads in cassettes instead of tissue.

Processing specimens

It is important to eliminate potential variables as far as possible when processing test and control groups of specimens. For example, the quality of processing reagents for each group should be identical. That is if you use fresh reagents for one group you must do so for the other. If you process at 45°C for the test group, do the same for the control (unless you are evaluating the effect of different temperatures during processing).

Embedding specimens

Specimens should be promptly embedded at the end of the processing run. As far as possible the time the specimens spend in wax prior to embedding should be standardized. The position of each specimen in the mould should be consistent because the orientation of a specimen to the microtome blade during microtomy has an effect on the ease of cutting and flattening sections and this forms part of the score generated.

Section cutting, mounting and drying

To assess the quality of a processed specimen in a paraffin block, we evaluate and compare important aspects of the section cutting process in generating a score for each section. It is therefore important to standardize all the steps in section preparation.

This includes:

- The temperature of the block when it is cut and the mode of cooling
- Section thickness
- Cutting speed, use of “huffing” (breathing on block face) to improve section quality (we prohibit this), length of ribbon
- The particular sections chosen for mounting from each ribbon (perhaps choose section three each time)
- The time each section spends on the water bath then, after mounting on a slide, the time spent draining before drying
- Drying time and temperature

Section staining

Poor processing, including the use of contaminated reagents, can influence the quality of staining in finished sections. This requires that the staining used on test and control sections must be standardized as far as possible so that valid comparisons can be made.

We have found that a good quality automated Hematoxylin and Eosin stain is satisfactory for our evaluations. If batches of sections are to be stained at different times it is essential to include a suitable control slide with each run so that run-to-run staining variations can be eliminated and staining problems due to processing properly identified.

Scoring sections

To prevent bias, all scoring is performed "blind" with the scorers being unaware of any details of the processing used on the specimens being examined. Processed specimen blocks are evaluated by scoring each block, and sections from each block, on 23 parameters in six groups. We use a simple three point scale for each parameter (2, 1, or 0, with zero being a fail) and calculate a percentage score for each of our six groups from the total score for each group. These percentages are added together and an overall percentage score is calculated. Despite the fact that there are different numbers of parameters in each group, each of our six parameter groups contribute equally to the final score (see Appendix for an example).

By using our six parameter groups as two groups of three, we are also able to generate separate scores for "section preparation and block storage", and "microscopic assessment" which we have found useful in comparative studies where very small differences exist between processing protocols.

The six groups of parameters are as follows:

Section preparation		
Group 1 (4 parameters)	Cutting	We assess the physical characteristics of each block and its compression and ribboning qualities
Group 2 (3 parameters)	Mounting	Here we assess the behavior of the section as it flattens on the waterbath
Block storage		
Group 3 (2 parameters)	Block stability on storage	After one week we examine the paraffin block for changes which may indicate incomplete processing
Microscopic assessment		
Group 4 (5 parameters)	Physical quality of section	We examine the physical quality of the section for indications that the tissue may not have been properly supported during cutting or incompletely processed
Group 5 (5 parameters)	Quality of tissue preservation	Careful and detailed microscopic examination is required to assess the quality of tissue preservation. A detailed knowledge of histology is needed to make this assessment. Appropriate critical features can be chosen depending on the specimen types being examined
Group 6 (4 parameters)	Quality of staining	Stained elements are assessed for definition and consistency

Table 5: Parameters for scoring blocks and slides for evaluation of processing

Our results are entered on an Excel spreadsheet. It should be noted that we provide scoring guidelines for each of the 23 parameters to assist our scorers in maintaining consistency (see Appendix). Our spreadsheets contain embedded formulae so that our scores are calculated automatically as we proceed. Our sheets contain space for twelve slides because we normally score at least six blocks of each tissue per processing run then derive a mean score for each tissue. An example of a complete score sheet is shown in the appendix. For simplicity, it shows only one set of scores not the usual twelve.

Score summary	Detail
1. Cutting	75.0%
2. Mounting	83.3%
3. Block stability on storage	75.0%
4. Physical quality of section	70.0%
5. Quality of tissue preservation	80.0%
6. Quality of staining (chemical)	75.0%
Section preparation & block storage (Groups 1+2+3)	77.8%
Microscopic assessment (Groups 4+5+6)	75.0%
Total Score (Groups 1+2+3+4+5+6)	76.4%

Table 6: Example of the score summary provided for each block and slide (The scores shown represent a block which we would consider to be of an acceptable standard but not of outstanding quality)

Results

We would consider any specimen which scored <50% (ie. zero on our three point scale) in any single parameter as a "fail" for that processing run, no matter what the total score. Any block and slide with a score of >80% is of high quality. This test procedure clearly identifies poor quality processing and processing artefacts.

Although you can use the scores achieved as a direct overall measure of processing quality, the system is better suited to direct comparisons of processing runs where we believe it can allow small differences to be objectively and reliably identified.

Discussion

It should be noted that in a scoring system such as this, or any system for evaluating tissue processing, the quality of tissue fixation is inevitably going to be reflected in the scores achieved. In fact, if the processing applied to specimens is completely standardized, this system can be used to evaluate fixation quality. This is why it is so important to standardize the fixation protocol as far as possible and to always process a control group with each processing test group.

Conclusion

The scoring system described in this paper has been used throughout the development of Peloris™. Although it is time consuming, we have found that it has allowed us to objectively and reproducibly evaluate the quality of specimens processed on Peloris and other processors. Using this system, we have been able to refine protocols and answer difficult questions about such things as the effects of reagent contamination on processing. We have found that anyone with a sound knowledge of histology and histological techniques can be rapidly trained to effectively use this scoring system.

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Appendix: Leica Microsystems score sheet showing details of parameters and scoring guidelines

Parameter	Scoring Guidelines			Score
Section preparation				
1. Cutting	**2**	**1**	**0**	
Texture of block	not brittle, homogenous, excellent texture	some brittleness, variation but cuttable	brittle, uneven, poor texture	1
Uniformity of block	complete uniform infiltration, well supported	some unevenness of infiltration	poor, uneven infiltration	2
Cohesiveness of block	no separation of components	some separation	severe separation	2
Ribboning & compression during cutting	sections ribbon, no compression	occasional breaks in ribbon, minor compression	sections detach, severe compression	1
				6/8 = 75.0%
2. Mounting	**2**	**1**	**0**	
Dehydration & clearing	no sweating	minor sweating	severe sweating	2
Cohesiveness	no separation of components	some separation of components	severe separation of components	1
Flattening	fl attens readily	flattens with some difficulty	impossible to flatten	2
				5/6 = 83.3%
Block Storage				
3. Block stability on storage (one week)	**2**	**1**	**0**	
Specimen shrinkage	no shrinkage	minor shrinkage	major shrinkage	1
Opacity	no change	some opacity has developed	considerable opacity observed	2
				3/4 = 75.0%
Microscopic Assessment				
4. Physical quality of section (excludes stain quality)	**2**	**1**	**0**	
disruption x4	no disruption	minor disruption, some holes or tearing	major disruption, holes, tearing	1
adhesion x4	completely flat	minor lifting	severe lifting	2
cracking (coarse - crazy paving) x4	no large cracks	some large cracks	severe large cracks	2
cracking (fine) x40	no cracks	some fine cracks	extensive fine cracks	1
section thickness	uniformly thin	some variation	extensive variation	1
				7/10 = 70.0%
5. Quality of tissue preservation	**2**	**1**	**0**	
nuclear detail (nucleolus, chromatin detail, nuclear envelope, vacuolation, shrinkage or swelling)	good	fair	poor	2
cytoplasmic detail (cohesive, uniformly preserved, texture shown, vacuolation, cell borders defined, swelling or shrinkage)	good	fair	poor	1
special features (kidney - basement membrane definition, liver - sinusoidal endothelium definition)	good	fair	poor	2
extracellular components and muscle (collagen, elastin)	good	fair	poor	1
uniformity of preservation (includes zonal fixation)	uniform across section	some variation	extreme variation	2
				8/10 = 80.0%
6. Quality of staining (chemical)	**2**	**1**	**0**	
uniformity	completely uniform	some variation	extreme variation	1
nuclear stain	strong and sharp, excellent	satisfactory	weak, poor definition, unsatisfactory	2
cytoplasmic stain	strong and sharp, excellent	satisfactory	weak, poor definition, unsatisfactory	1
extracellular components & muscle (collagen, elastin)	strong and sharp, excellent	satisfactory	weak, poor definition, unsatisfactory	2
				6/8 = 75.0%
			Total/46	**35**

This score sheet shows the results for a single H&E slide which we would consider to be of an acceptable standard but not of outstanding quality.

Score Summary	
Cutting	75.0%
Mounting	83.3%
Block stability on storage	75.0%
Physical quality of section	70.0%
Quality of tissue preservation	80.0%
Quality of staining (chemical)	75.0%
Section prep & block storage (1+2+3)	77.8%
Microscopic assessment (4+5+6)	75.0%
Total Score	**76.4%**

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