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Comparison of Varying Tissue Freezing Methods on Murine Colonic Tissue

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Comparison of Varying Tissue Freezing Methods on Murine Colonic Tissue

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By

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Abstract:

Histology often requires a tissue specimen to be embedded so that it may be sectioned, stained, and mounted on a microscope slide for viewing. One common method of tissue embedding for rapid histology is freezing, since freezing allows tissue to be stored without the need for fixing. Frozen tissue is often embedded in a medium such as Optimal Cutting Temperature (OCT) compound so that it can be sectioned using a cryostat. However, factors such as ice-crystal formation during the freezing process can cause damage to the tissue. As such, the protocol used to freeze the tissue can affect the quality of the slides.

The purpose of this project is to compare different freezing methods and examine their strengths and weaknesses when applied to murine colonic tissue. Murine colonic tissue was frozen using two snap-freezing methods, piezoelectric freezing, and two different cold storage methods, each with their own three to four variations. Transverse sections were made in a cryostat, which were mounted on slides and stained using a hematoxylin and eosin (H&E) staining protocol. The sections were then imaged using a light microscope. A blind test was conducted to rate the image quality and inter-rater agreement was calculated using Fleiss's Kappa. Paraffin embedding obtained the highest score, while OCT embedding inside a -80°C freezer received the second highest score.

Comparison of Varying Tissue Freezing Techniques on Murine Colonic Tissue.

Background:

This project was originally conceived due to the goals of a parent project. The parent project sought to examine the change in collagen morphology in colonic tissue as dysplasia progresses. A large part of the project is histological validation of changes in collagen structures, and one of the most important steps in the preservation of these structures in the histological process is the embedding of tissue. Tissue embedding provides thin, delicate tissue with additional bulk in order to keep rigidity during slicing and preserves the tissue for storage. Embedding can be performed in three primary ways: embedding in an acrylic resin, embedding in Paraffin wax, and embedding via freezing in a medium. For the purposes of this project, resin embedding will not be discussed, as it requires a specialized microtome and diamond or glass blades. However, paraffin-embedding and freezing are both far more common methods of embedding tissue.

Paraffin embedding is carried out by encasing a fixed tissue sample in a block of Paraffin wax. Paraffin is effective for preservation of tissue morphology, proteins, and nucleic acids. (1) Paraffin embedded sections can even be stored at room temperature until they are sectioned. However, the Paraffin embedding process spans multiple days, as it requires the tissue to be fixed, anywhere from several hours to overnight, and then dehydrated prior to wax infiltration.

Frozen embedding of tissue is carried out by freezing tissue in a medium, such as optimal cutting temperature compound (OCT). Freezing can be performed using liquid nitrogen, dry ice, piezoelectric freezing, and other methods. Histology using frozen sections requires a greatly reduced amount of time. Tissue can be obtained and immediately frozen, sectioned, and stained, all within the span of a day. (2) Of course, freezing also has several disadvantages. Formation of

ice crystals in frozen tissue can result in damage to tissue morphology. (3) The changing temperatures involved in freezing tissue can also result in compromised morphology due to osmosis. (4)

When it comes to the quality of the images, paraffin embedding is the best choice for the parent project's goals. However, it may be possible to utilize a freezing method that can still provide useful data, despite a reduced quality, for the project. The ideal frozen embedding protocol would be a method that minimizes damage to tissue morphology. In the case of murine colonic tissue, where relevant structures lie in the top 100-200 microns of exposed epithelial layer, small differences in freezing protocol can have a big effect on image quality. In this project, the intent is to explore different tissue freezing protocols and determine the resulting advantages and disadvantages to each method. The sheer variety of freezing methods will allow for examination of how each method affects the quality of the image obtained and determination of the optimal methods to achieve images with qualities that are relevant to the histological purposes.

Project Aims:

The purposes of this study are as follows:

Aim 1: Optimization of in-house sectioning of murine colonic tissue, and slide preparation for H&E staining

Aim 2: Qualitative comparison of quality in H&E–stained sections after various freezing protocols

Experimental Methods:

Aim 1: Optimization of in-house sectioning

FREEZING METHOD	VERSION:				
SNAP FREEZE	JoVE (Hank's)	JoVE (Hank's - cold)	JoVE (PBS)	Pre-treated (95% EtOH)	
SNAP FREEZE (DRY)	Baby powder	Cotton swab	Filter paper		
PIEZOELECTRIC	Straight (petri)	Insulated (s. box)		Pre-treated (95% EtOH)	
COLD STORAGE (-80)	Straight (petri)	Insulated (s. box)		Pre-treated (95% EtOH)	
COLD STORAGE (-20 TO -80)	Straight (petri)	Straight (-20) then Insulated (-80)	Insulated (s. box)	Pre-treated (95% EtOH)	

Table 1: List of freezing methods with their different variations. This list contains all the attempted methods.

Colon tissue was excised from a nude athymic mouse provided by Dr. Muldoon's lab, and prepared for freezing using 3 different methods: snap freezing, cold storage, and piezoelectric freezing. With each method several variations in procedure were attempted.

For snap freezing, tissues were directly freezing prior to embedding using isopentane chilled by liquid nitrogen. (5) (6) The tissue was frozen indirectly using isopentane, rather than directly in liquid nitrogen, to ensure rapid freezing. Because nitrogen is a gas at room temperature, direct contact with the unfrozen tissue will cause the liquid nitrogen to evaporate, resulting in a gaseous barrier between the liquid nitrogen and the tissue. This gaseous barrier will insulate the tissue, slowing the freezing process. As such, liquid isopentane that has been chilled by liquid nitrogen is used. After dissection, fixation was performed on four tissue samples with a different fixative for each piece: room temperature Hank's Buffer, cold Hank's Buffer, phosphate-buffered saline (PBS), and 95% ethanol (EtOH). The four samples were each fixed for 10 minutes. Three more tissue samples were dried using three different methods. One tissue sample was covered in baby powder, another dried using a cotton swab, and another dried using filter paper. In preparation for freezing, a specialized bowl was filled with liquid nitrogen. A small cup was then made of

aluminum foil and filled with isopentane. The cup was placed inside the bowl, and the isopentane was allowed to chill to its melting point. Once the tissues were fixed or dried, they were each sandwiched between two dull razorblades. The blades kept the tissue flat while also providing a surface to grip with forceps, without gripping and potentially damaging the tissue. After freezing, each of the tissue-blade sandwiches were wrapped in aluminum foil to prevent desiccation (7) and stored in the -80°C freezer.

For the cold storage freezing tissue samples in cryomolds were frozen directly inside the storage freezers. The attempted tests for this tissue were to judge the effects of differing freezing rates on the tissue. Four of the tissue samples were fixed in 95% EtOH for 10 minutes. These four tissue samples, as well as four unfixed tissue samples, were then embedded in OCT. The samples were placed in cryomolds, which were then filled with OCT. A normal sample and an EtOH sample were then paired for each of the variations. Two pairs were placed into the -80°C freezer, one directly inside the freezer and another inside a Styrofoam box. The other two pairs were then placed into the -20°C freezer, one directly and the other in a Styrofoam box. The uninsulated pair was moved into the -80°C freezer after 30 minutes and the insulated pair was moved after 45 minutes.

For piezoelectric freezing, two normal samples and two EtOH dehydrated samples were used. After the samples were embedded in the OCT cryomolds, piezoelectric freezing was performed in the cryostat using the PE freezing plate. The samples were then stored in the -80°C freezer, with one normal and one EtOH sample in a Styrofoam box.

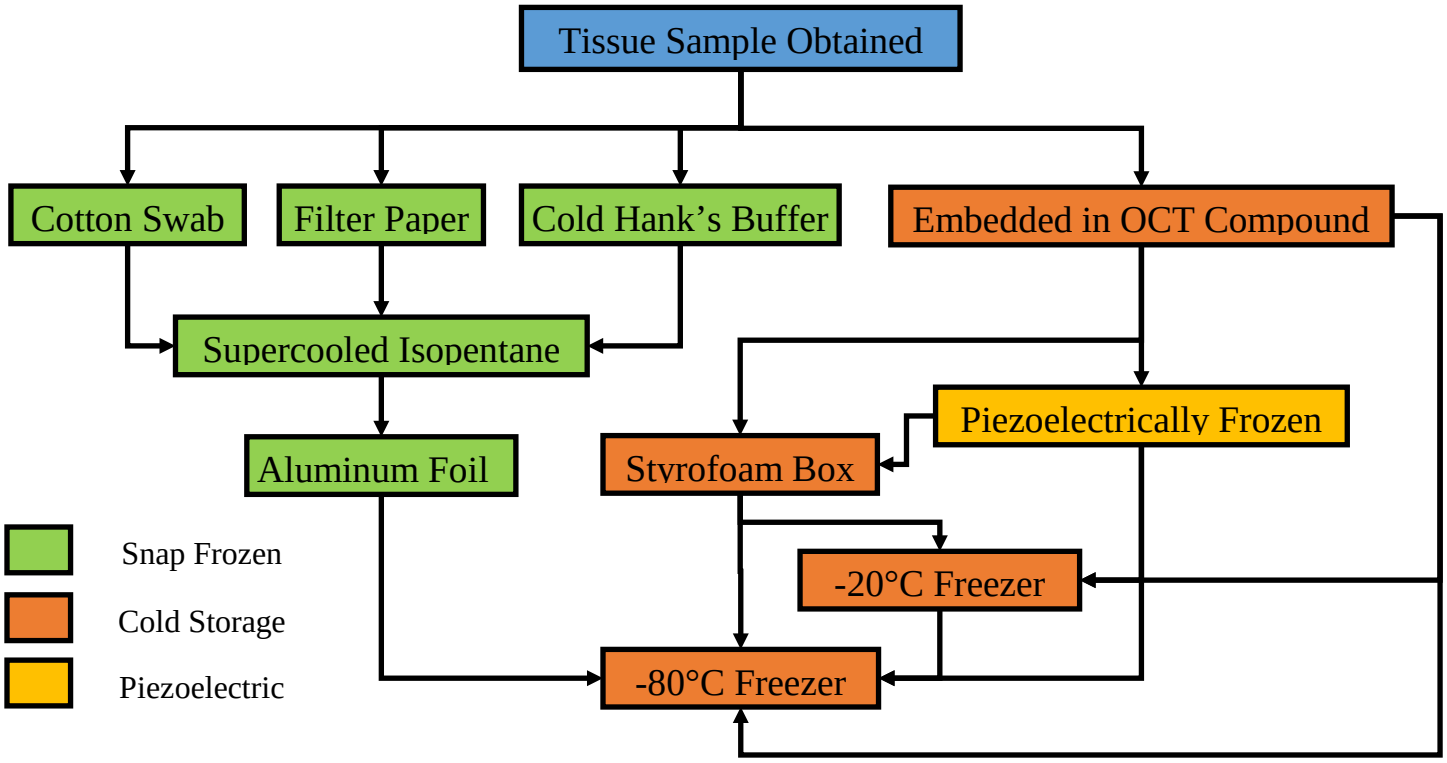


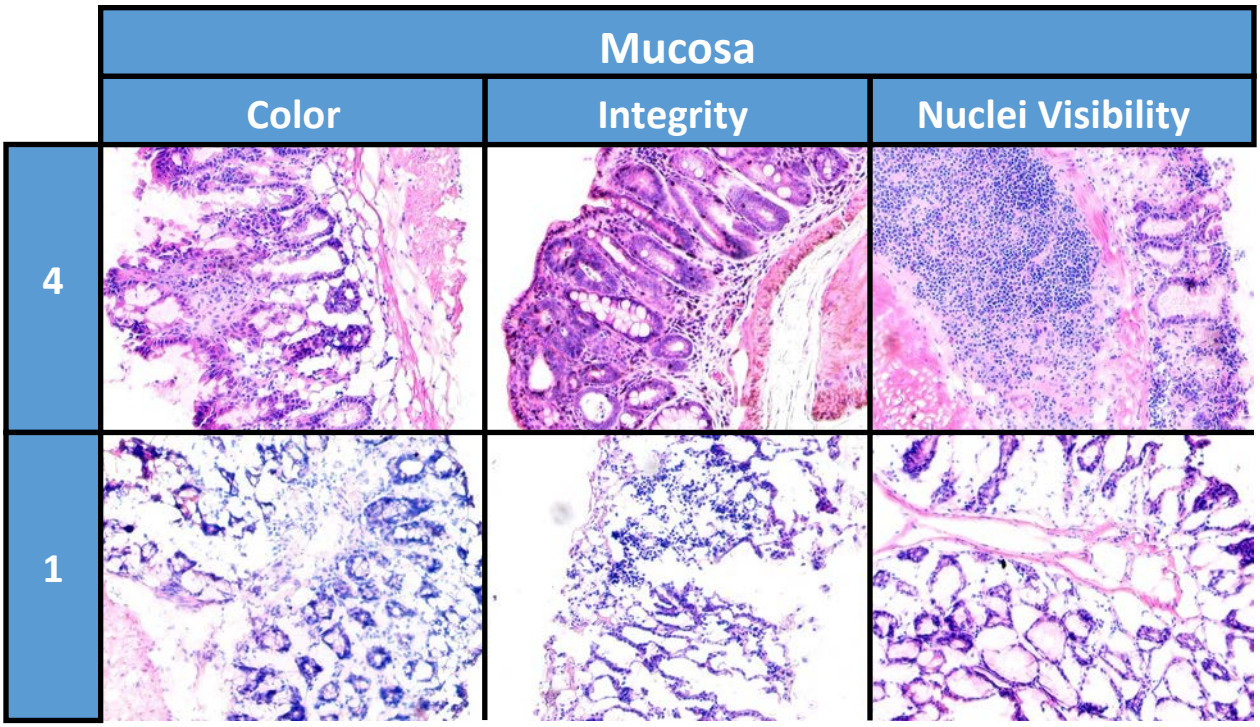
Figure 1: Flow chart for tissue freezing. This chart only illustrates the samples from which images were obtained.

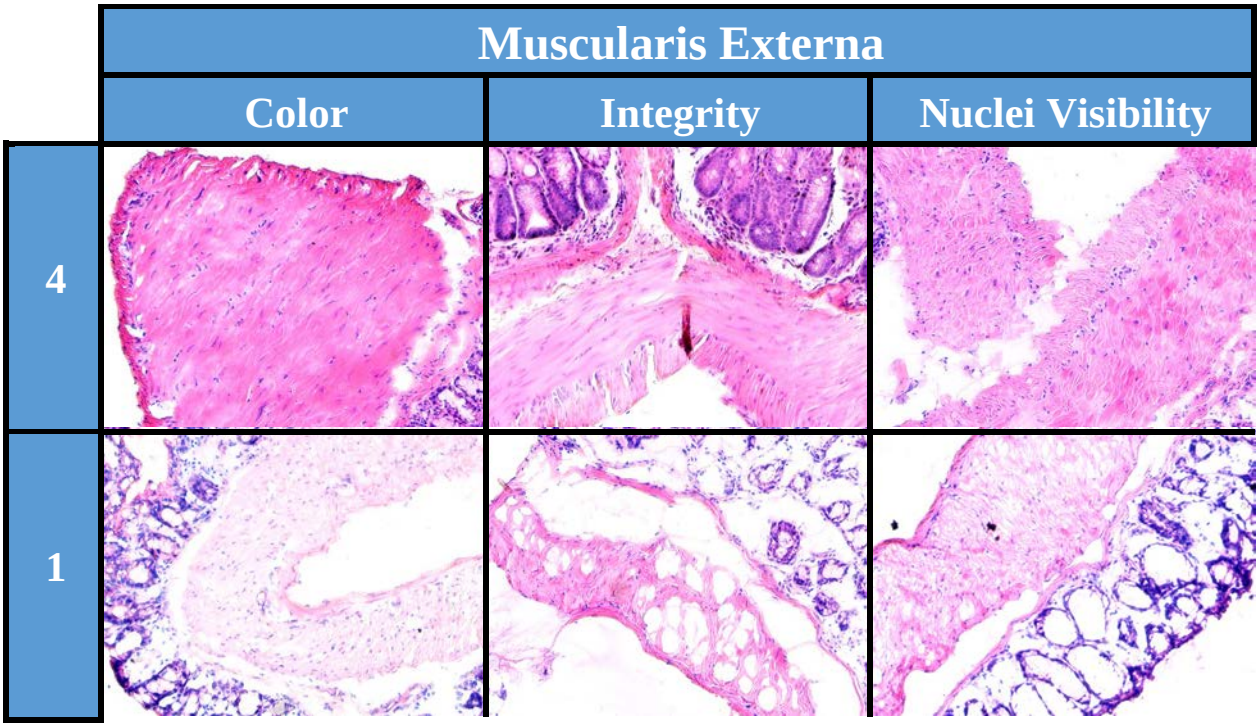
The snap frozen tissues had been frozen and stored without being embedded, so they were embedded prior to sectioning. The samples were each placed as flat as possible onto pre-frozen blocks of OCT, then covered in a new layer of OCT and allowed to freeze in the cryostat. Then the tissues were sectioned at a thickness of 5 microns, the sections were mounted on slides, and the slides were stored in a slide box in the -80°C freezer. The slides were stained simultaneously using hematoxylin and eosin (H&E), to ensure that any variations in color be unrelated to staining time. H&E was chosen as the stain because it is considered the golden standard of stains, with hematoxylin binding to nucleic acids and eosin binding to general proteins. This provides a general overview of the most important structures in the tissue, without being overly specific. Finally, the images were obtained using a light microscope. After a paraffin embedded H&E section was used to calibrate the microscope, three images were taken of each slide at 10x magnification.

Aim 2: Determination of quality in H&E sections

The images were graded in two main categories: the two tissue layers, the mucosa and the muscularis externa. These two tissue layers were graded for three qualities: color, integrity, and nuclei visibility, for a total of six categories. After sectioning, a rubric was devised by which the images would be given scores in each category from one to four, four being the highest. The rubric included guidelines for what each score represents, as well as examples of high-quality and low-quality images. (Appendix A) The rubric workbook was given to three graduate students in Dr. Muldoon's lab, along with the obtained images without labels. These graduate students were chosen as raters for their familiarity with H&E staining and murine colon tissue images. The images were unlabeled in order to prevent any bias associated with preconceptions the raters may have about the different freezing methods. Included in the unlabeled images was a paraffin embedded sample, to act as a control.

Each tissue sample was represented by three images. Each sample was given nine scores in each of the six categories, three image scores by three rater scores. The average score was obtained for each category, then these scores were averaged again to obtain overall average scores for each tissue sample.





Tables 2 & 3: Scoring system examples. Images shown received the maximum (4) and minimum (1) scores in their given categories.

Results and Discussion:

Tissue Section Quality

Of the attempted freezing methods, only nine were sufficiently undamaged to provide slides that could be imaged and graded. Three images were obtained for each sample.

Average Scores	*Mucosa C*	*Mucosa I*	*Mucosa N*	*Muscularis C*	*Muscularis I*	*Muscularis N*	***Average***
PARF	4	4	4	4	4	3	3.69
Ins 20	2	1	2	3	2	3	2.43
Ins 80	2	1	2	3	2	3	2.46
CHB	1	1	2	1	1	2	1.44
CS	2	2	2	3	2	3	2.28
FP	4	2	4	3	2	3	3.09
PEI	2	3	2	2	3	2	2.17
PES	2	2	2	3	3	4	2.80
Str 20	2	2	2	2	2	2	1.96
Str 80	3	2	3	4	3	4	3.20

Table 4: Average scores for each sample. The average scores for each category are the (rounded) averages of nine values; three images per sample by three raters. The scores for each category were then averaged to obtain an overall score for each

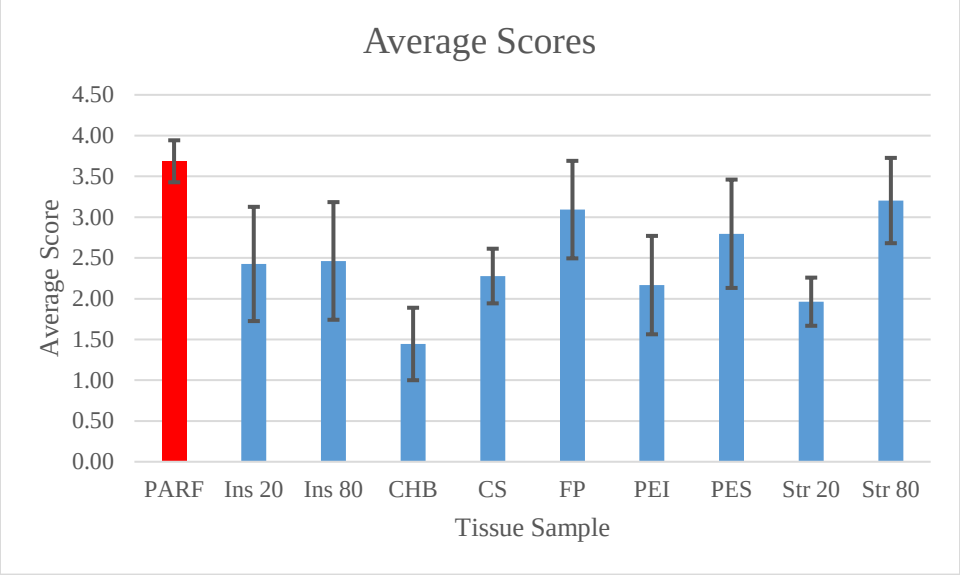


Figure 2: Bar chart of the average scores for each sample. The red bar indicates the paraffin embedded control sample.

As was expected, the paraffin embedded sample received the highest score at 3.69. It received the highest quality ratings in all but the muscularis nuclei visibility category. Of the nine

frozen samples, the sample with the highest score was the Straight -80°C sample, at a score of 3.20. This is significant because this method is also the fastest and simplest method. It only involves placing the tissue sample into a cryomold, filling the cryomold with OCT, and then placing the cryomold directly into the -80°C freezer for storage. The lowest scoring tissue sample was the sample treated with cold Hank's buffer prior to snap freezing, with a score of 1.44.

This low score stands in contrast to the Filter Paper sample, which while also being snap frozen received the second highest score of the nine samples at 3.09. As mentioned above, the snap frozen samples were frozen onto blocks of OCT prior to sectioning by covering them in a new layer of OCT. When the samples were sectioned transversely, it was observed that a sort of "fault line" existed between the OCT block and the new layer of OCT, with the tissue sample right in the middle. While this fault line is only just visible to the naked eye, it results in added stresses at the microscopic level of cryosectioning. It seems that this fault line resulted in some sectioning complications for the tissue samples that were not embedded in the OCT blocks beforehand.

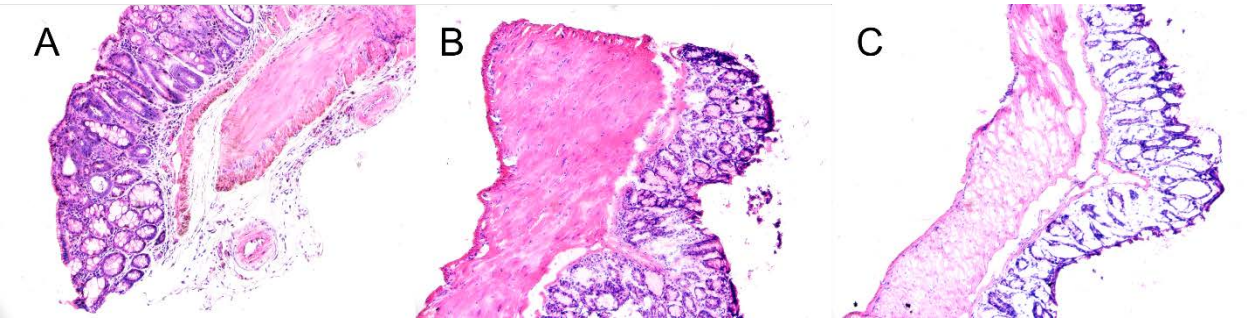


Figure 3: 3 examples of the obtained images. (A) Paraffin embedded tissue, used as the golden standard for this study. (B) Straight -80°C image, which obtained the second highest total score. (C) Straight -20°C image, which received a mediocre score.

Statistical Analysis

Statistical analysis was performed in MATLAB. (Appendix B) Inter-rater agreement was calculated using Fleiss's Kappa. Fleiss's Kappa was chosen because it compares the agreement between three or more raters with the probability of the raters agreeing by chance. (8)

$$\kappa = \frac{\bar{P} - \bar{P}_e}{1 - \bar{P}_e}$$

Equation 1: Fleiss's Kappa

Inter-rater reliability serves to main purposes: to support the scores that were consistently chosen between the three raters, and to prove the efficacy of the raters' training. A high kappa value indicates an increased consistency between the raters, and by extension an increased ability of the raters to identify defects in the rated sample. (9) Training the raters effectively will improve these factors, and effective training will be represented in an increased kappa value.

The data was first converted into a binary system of acceptable scores vs unacceptable scores. This was done because Fleiss's kappa treats the data as nominal, so information is lost in ordered-categorical rating systems. The 1-4 rating scale is ordered-categorical, where order of the categories matters (two follows one, three follows two, etc.). However, this problem can be circumvented by applying Fleiss's kappa to a binary scale. Scores of one and two were converted to zeros, while scores of three and four were converted to ones. Kappa values were then calculated for each of the six categories, and then the six kappa values were averaged to obtain an overall kappa.

Category	Mucosa C	Mucosa I	Mucosa N	Musc C	Musc I	Musc N
Kappa	0.3304	0.4000	0.8500	0.7000	.4570	0.2547
Average Kappa: 0.4987						

Table 5: Individual kappa values for each of the six categories and the average kappa, calculated from a binary score distribution.

Kappa values varied over the six categories, ranging from fair agreement at 0.2547 to near-perfect agreement at 0.8500. The overall kappa value is 0.4987, which is considered moderate agreement. (10)

Conclusions and Future Directions:

Based on the acquired data, the recommended freezing method would be embedding in OCT directly in a -80°C freezer. The Straight -80°C sample received the highest score of the freezing methods, which is supported by a high level of inter-rater agreement. It is also among the simplest methods, in which the sample is frozen in the same freezer where it will be stored. While it does not allow for immediate sectioning, it does allow freezing to take place overnight. The sample is also embedded in the OCT while it is frozen, allowing for easy transverse or en face sectioning. For same-day sectioning, drying the sample with filter paper before freezing in isopentane would be recommended. However, the tissue must be embedded in OCT before sectioning, resulting in potential complications when sectioning transversely. Piezoelectric freezing is still the fastest method and allows for the tissue to be fully embedded in an OCT block. Sectioning instantly after freezing would also improve the score, as storage allows vitreous ice in the tissue sample to slowly crystallize, causing further damage to the tissue. (11) Paraffin embedding received the best score. It is the best choice for sensitive data, or simply for attaining the highest quality images possible.

Further studies would be needed to minimize damage caused by cryosectioning. While transverse sectioning guarantees the presence of multiple tissue layers in a slide, sectioning en face would eliminate the “fault line” variable present in the snap-frozen tissue sections, while also simplifying the cryosectioning procedure. Staining with other types of tissue stain would highlight

different parts of the tissue. Masson's Trichrome would allow for better analysis of the collagen fibers in the tissue samples.

Increasing the number of raters would improve the accuracy and precision of the Fleiss's Kappa statistic, which could further bolster or confute the conclusions drawn from the data. Analysis of individual tissue samples using Fleiss's kappa would allow for individual agreement of the raters to be assessed on a per-sample basis. For example, a high kappa value for the paraffin embedded sample would indicate that the raters had a high level of agreement on the score. This would mean that there was little ambiguity in the sample; it clearly deserved the score it was given. However, Fleiss's kappa can be prone to paradox. (12) Fleiss's kappa encounters a problem here, as it is unequipped to deal with perfect agreement between multiple samples. For example, if the samples are given the exact same rating by every rater, then that rating was chosen 100% of the time in the data set. While this indicates a perfect level of agreement between the raters for the samples, Fleiss's kappa calculates a standard error (P_e) of 1, or a 100% chance that the raters would agree by chance. As can be seen in Equation 1, this results in an irrational number, as Fleiss's kappa equation attempts to divide by zero. For this analysis to be attempted, it may be necessary to use a statistical measure other than Fleiss's kappa. If it were to be done, it would reinforce the scores of the individual tissue samples.

Acknowledgements:

Special thanks to: Gage Greening, Cassandra Reed, and John Kim.

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Comparison of Varying Tissue Freezing Methods on Murine Colonic Tissue

IMAGE ASSESSMENT WORKBOOK:

THE PURPOSE OF THIS WORKBOOK IS TO PROVIDE A RUBRIC AND A FRAME OF REFERENCE FOR THE BLIND ASSESSMENT OF H&E STAINED MOUSE COLON IMAGES.

Rubric (Mucosa)

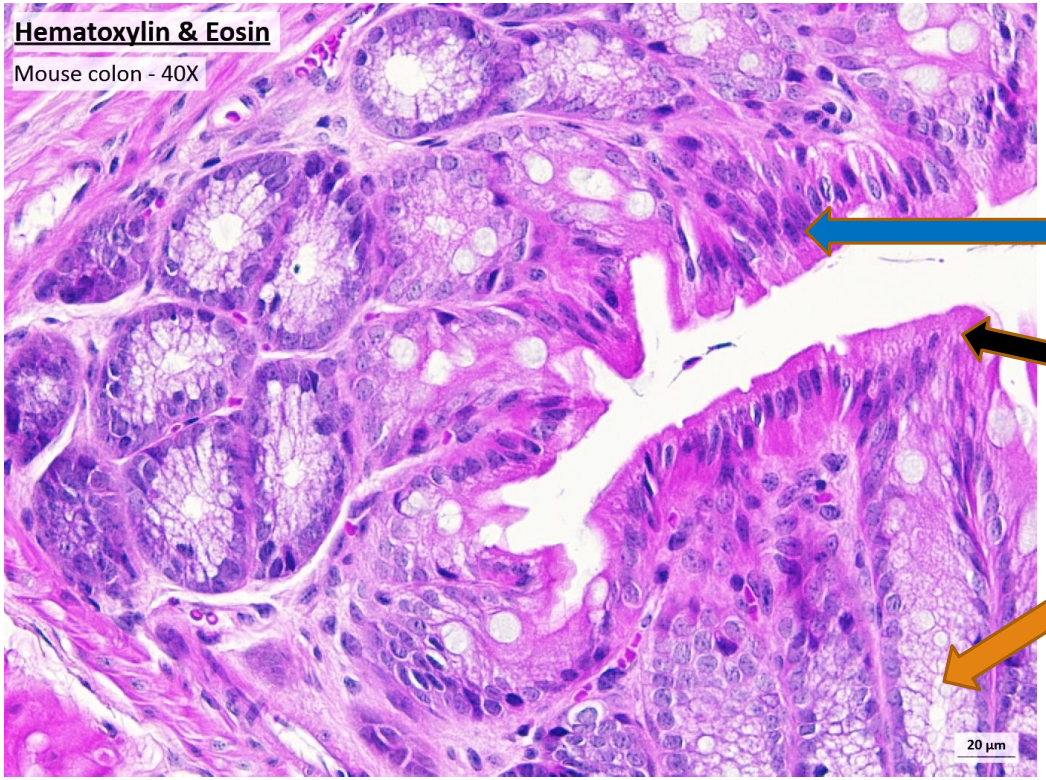
Tissue Type	Category	1	2	3	4
Mucosa	**Coloration/Shade**	Extremely dark purple (or no purple at all)	Dark, uniform violet, no discernable pink	Primarily dark purple, with some pink shading	Dark violet nuclei, pink shade highlights microstructures
	Integrity	Complete destruction of crypt structures	Visible damage to the crypt structures themselves	Some gaps between crypt structures	Crypts are tightly interlocked, no visible damage
	Nuclei Visibility	Nuclei are undetectable	Nuclei are barely detectable	Most nuclei can be differentiated from the mucosa	Nuclei are clearly and easily discernable

Appendix A

Rubric (Muscularis Externa)

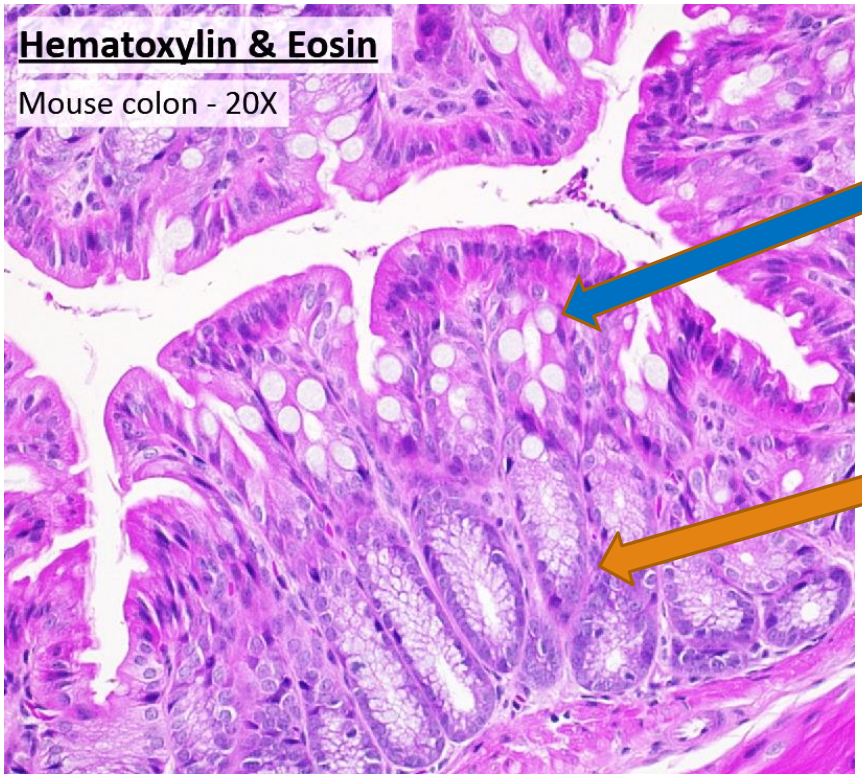
Tissue Type	Category	1	2	3	4
Muscularis Externa	**Coloration/Shade**	Almost completely invisible (or purple)	Extremely weak shade of pink	Good shade of pink, violet nuclei	Vibrant shade of pink, visible muscle fiber orientation, violet nuclei
	Integrity	Complete destruction	Large, gaping holes	Smattering of small holes	Virtually no damage
	Nuclei Visibility	Nuclei are undetectable	Nuclei are visible, but difficult to differentiate	Nuclei are visible and discernable	Individual nuclei are clearly discernable

Example: Mucosa Coloration



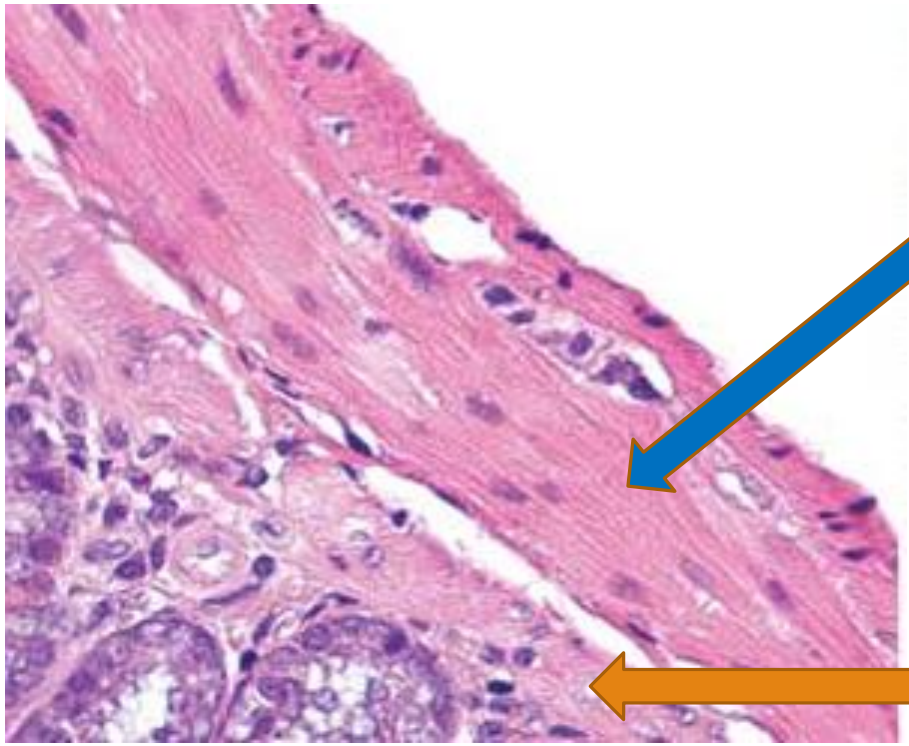
- Varying shades of purple.
- Dark purple nuclei.
- Area between crypts is a pink shade (can be less intense than this example).
- Cells within crypt are visible, with what appears to be a pale violet webbing.

Example: Mucosa Integrity



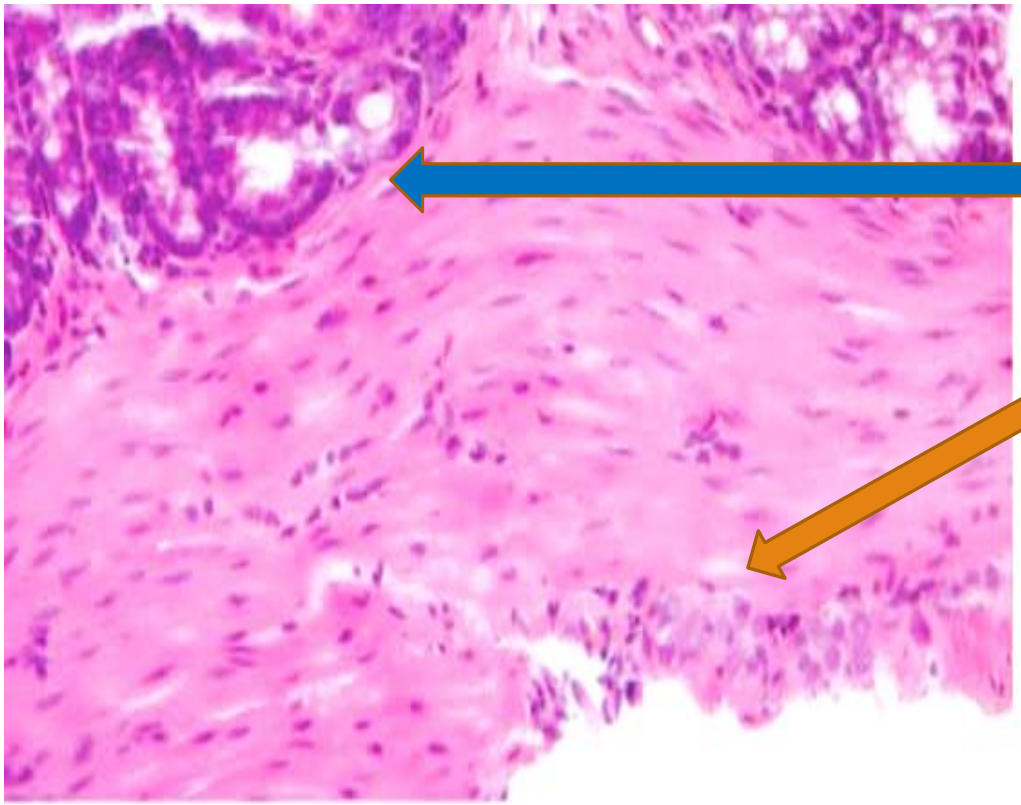
- The white circles are not ice damage.
- Gaps between crypt structures are filled by pink collagen, not white holes.

Example: Muscularis Externa Coloration



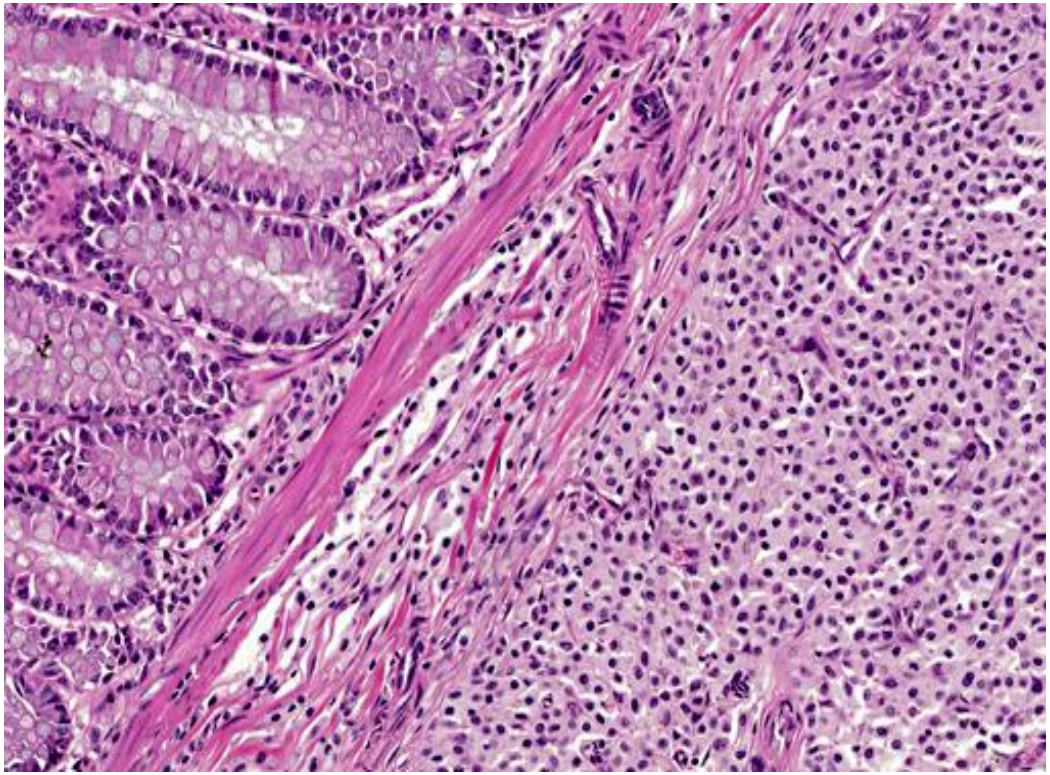
- The muscularis externa is a bright pink color.
- Dark purple nuclei.
- Visible muscle fiber orientation.
- Basal Membrane is a slightly different shade.

Example: Muscularis Externa Integrity



- Minimal separation between mucosa and muscularis.
- Minimal white spots.
  - Rarely will there be none.

Example: Nuclei Visibility



- Dark violet nuclei
- Easy to differentiate between individual nuclei.

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Fleiss's Kappa Calculation

James Hughes And Sandra Gordon April 4,2018

```
%  Kappa stuff pt2

clear all
clc
```

Scores and averages

```
Scores = [4 4 4 4 3 4 4 3 4 4 4 3 4 4 4 3 2 3
4 4 3 3 3 4 4 3 4 4 4 4 4 4 4 3 4 3
4 4 3 4 4 4 4 3 4 4 4 3 4 4 4 4 3 4
3 2 3 2 1 3 1 2 3 3 3 3 3 2 4 3 3 3
3 2 2 1 1 2 2 2 2 3 4 4 2 2 2 3 4 3
2 2 3 1 1 1 3 2 2 3 4 3 1 2 1 2 4 3
2 3 1 1 1 1 2 2 2 3 2 4 2 3 3 3 3 4
2 2 2 2 1 2 2 2 1 3 4 4 2 2 2 2 4 3
3 3 3 2 1 2 2 2 3 3 3 4 2 3 3 4 3 3
1 1 2 1 1 1 2 2 3 1 1 1 1 1 2 1 1 3
1 1 2 1 1 2 2 1 2 1 1 1 1 1 1 2 2 3
1 1 2 1 1 1 2 2 2 1 1 1 1 1 2 1 2 3
2 2 3 2 2 3 2 2 3 3 3 4 1 2 4 2 2 3
3 1 2 2 2 2 2 3 2 2 1 3 1 1 3 3 3 2
3 2 3 2 1 2 3 2 2 2 3 3 1 1 2 3 2 3
4 3 4 3 2 3 4 3 4 3 4 4 3 3 3 4 3 4
3 3 4 1 1 2 4 3 4 3 2 3 2 2 1 3 3 4
4 3 4 3 2 3 3 3 4 3 4 4 2 3 3 3 3 4
2 1 1 3 2 4 2 1 1 2 1 2 3 3 4 3 2 2
3 2 2 3 2 1 3 2 2 2 1 2 3 3 4 3 2 2
2 1 1 3 2 3 2 1 1 2 1 2 2 2 4 3 2 2
3 1 2 3 2 3 3 2 3 3 3 3 3 3 4 4 3 3
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2 1 2 3 2 2 2 1 2 3 3 3 2 3 4 3 3 3
2 1 2 2 1 3 2 1 2 2 1 3 2 2 4 2 2 3
3 1 1 1 1 1 3 1 1 1 1 2 1 1 1 2 1 2
4 2 3 3 2 4 3 3 3 4 4 4 4 3 4 4 3 4
3 3 3 3 2 3 4 3 3 4 4 4 3 3 4 3 3 4
4 1 3 2 1 2 3 2 3 4 3 4 3 3 4 4 3 4
];
```

```
[slidecount,categoryAndRatercount] = size(Scores);
% Average per sample (3 slides)

newAvgScores = [];
for i = 1:3:slidecount

sample = Scores(i:i+2,:);
subAverage = mean(sample);
newAvgScores = cat(1,newAvgScores, subAverage);

end

newAvgScores = round(newAvgScores);
newAvgScores(newAvgScores<=2)=0;
newAvgScores(newAvgScores>=3)=1;

% Reduce scores to binary
Scores(Scores<=2)=0;
Scores(Scores>=3)=1;
```

Calculating Stat Distribution

```
%Counting how many per rate (0-1)
sMuCo = zeros(10,2);
for i = 1:10

%count for each sample, across 2 ratings
    stat1 = sum(newAvgScores(i,1:3)==0);
    sMuCo(i,1) = stat1;

    stat2 = sum(newAvgScores(i,1:3)==1);
    sMuCo(i,2) = stat2;
end

sMuInt = zeros(10,2);
for i = 1:10

%count for each sample, across 2 ratings
    stat1 = sum(newAvgScores(i,4:6)==0);
    sMuInt(i,1) = stat1;

    stat2 = sum(newAvgScores(i,4:6)==1);
    sMuInt(i,2) = stat2;
end


sMuNu = zeros(10,2);
for i = 1:10

%count for each sample, across 2 ratings
    stat1 = sum(newAvgScores(i,7:9)==0);
    sMuNu(i,1) = stat1;
```

```
    stat2 = sum(newAvgScores(i,7:9)==1);
    sMuNu(i,2) = stat2;
end


sMusCo = zeros(10,2);
for i = 1:10

%count for each sample, across 2 ratings
    stat1 = sum(newAvgScores(i,10:12)==0);
    sMusCo(i,1) = stat1;

    stat2 = sum(newAvgScores(i,10:12)==1);
    sMusCo(i,2) = stat2;
end

sMusInt = zeros(10,2);
for i = 1:10

%count for each sample, across 2 ratings
    stat1 = sum(newAvgScores(i,13:15)==0);
    sMusInt(i,1) = stat1;

    stat2 = sum(newAvgScores(i,13:15)==1);
    sMusInt(i,2) = stat2;
end

sMusNu = zeros(10,2);
for i = 1:10

%count for each sample, across 2 ratings
    stat1 = sum(newAvgScores(i,16:18)==0);
    sMusNu(i,1) = stat1;

    stat2 = sum(newAvgScores(i,16:18)==1);
    sMusNu(i,2) = stat2;
end
```

Run fleiss for overall Kappa

```
kappaMuCo = fleissnew(sMuCo)
kappaMuInt = fleissnew(sMuInt)
kappaMuNu = fleissnew(sMuNu)
kappaMusCo = fleissnew(sMusCo)
kappaMusInt = fleissnew(sMusInt)
kappaMusNu = fleissnew(sMusNu)

kappaarray = [kappaMuCo kappaMuInt kappaMuNu kappaMusCo kappaMusInt
 kappaMusNu];
avgKappa = mean(kappaarray)


kappaMuCo =
```

```
    0.3304

kappaMuInt =

    0.4000

kappaMuNu =

    0.8500

kappaMusCo =

    0.7000

kappaMusInt =

    0.4570

kappaMusNu =

    0.2547

avgKappa =

    0.4987
```

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```
function k = fleissnew(x,varargin)
% FLEISS: compute the Fleiss'es kappa
% Fleiss'es kappa is a generalisation of Scott's pi statistic, a
% statistical measure of inter-rater reliability. It is also related
to
% Cohen's kappa statistic. Whereas Scott's pi and Cohen's kappa work
 for
% only two raters, Fleiss'es kappa works for any number of raters
 giving
% categorical ratings (see nominal data), to a fixed number of items.
 It
% can be interpreted as expressing the extent to which the observed
 amount
% of agreement among raters exceeds what would be expected if all
 raters
% made their ratings completely randomly. Agreement can be thought of
 as
% follows, if a fixed number of people assign numerical ratings to a
 number
% of items then the kappa will give a measure for how consistent the
% ratings are. The scoring range is between 0 and 1.
%
%           Created by Giuseppe Cardillo
%           giuseppe.cardillo-edta@poste.it
%
% Modified by James Hughes and Sandra Gordon
%
% To cite this file, this would be an appropriate format:
% Cardillo G. (2007) Fleiss'es kappa: compute the Fleiss'es kappa for
 multiple raters.
% http://www.mathworks.com/matlabcentral/fileexchange/15426

%Input Error handling
p = inputParser;
addRequired(p,'x',@(x) validateattributes(x,{'numeric'},
{'nonempty','integer','real','finite','nonnan','nonnegative'}));
addOptional(p,'alpha',0.05, @(x) validateattributes(x,{'numeric'},
{'scalar','real','finite','nonnan','>',0,'<',1}));
parse(p,x,varargin{:});
x=p.Results.x; alpha=p.Results.alpha;
clear p
n=size(x,1); %subjects

%check if the raters are the same for each rows
r=sum(x,2);
if any(r-max(r))
    error('The raters are not the same for each rows')
end

m=sum(x(1,:)); %raters
a=n*m;
pj= zeros(1,2);
pj(1,1)=(sum(x(:,1))./(a)); %overall proportion of ratings in category
 j
```

```
pj(1,2)=(sum(x(:,2))./(a));

% we replaced the kappa with our own equation...
% pa uses x, or main input, and m
% m is number of raters/people
[rows,~] = size(x);
m = 3;
pa = (sumsqr(x)-3*rows)/(3*rows*(3-1));
pe = sumsqr(pj);
k = (pa-pe)/(1-pe);
end
```

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