TACMASR

Embedding Formalin-Fixed Tissue

A. Place tissue in 65 °C oven
    a. Hnk;szerhg
    b. Rfh[pgerh
B. Open cassette to reveal tissue on 65 °C warmed workspace
C. Select mold that will allow at least 3 mm of space around the tissue
D. Place tissue into the pre-heated mold and orient
    a. Largest surface area of tissue oriented onto the mold
    b. Tissue with layering such as skin or intestine will be placed on its edge
E. Dispense 65 °C paraffin into the mold
F. Hold the tissue in place and move towards the cold plate
G. When the bottom of the mold has hardened paraffin, place the cassette top over the mold
H. Top off the cassette-mold with more paraffin
I. Rest on cold plate for 45 minutes before snapping the hardened paraffin-cassette from the mold
J. Deparaffinize edges of cassette with heated Para-Trimmer

Hematoxylin and Eosin Staining

A. After slides have been sectioned and dried, place onto staining rack and into 65ºC oven for 20 minutes
B. Remove slides from oven and allow them come to room temperature for 5 minutes
C. Dip slide rack into xylene 10 times then leave the rack sitting in the xylene for 3 minutes
D. Repeat in 2 more xylene changes
E. Dip slide rack into 100% ethanol 10 times then leave the rack sitting in the ethanol for 3 minutes. Repeat into one more 100% ethanol.
F. Dip slide rack into 95% ethanol 10 times then leave the rack sitting in the ethanol for 3 minutes
G. Dip slide rack into 70% ethanol 10 times then leave the rack sitting in the ethanol for 3 minutes
H. Place slides under running water for 30 seconds
I. Switch recipient and place slides under running water for 30 additional seconds
J. Dip slide rack into hematoxylin 10 times then leave the rack sitting in the hematoxylin for 2 minutes and 30 seconds
K. Run slides under water for 1 minute
L. Dip slide rack into clearview 10 times then leave the rack sitting in the clearview for 30 seconds
M. Run slides under water for 30 seconds
N. Dip slide rack into bluing 10 times then leave the rack sitting in the bluing for 30 seconds
O. Run slides under water for 30 seconds
P. Dip slide rack into 70% ethanol 10 times then leave the rack sitting in the ethanol for 1 minute
Q. Dip slide rack into eosin 10 times then leave the rack sitting in the eosin for 2 minutes and 30 seconds
R. Dip slide rack into 100% ethanol 10 times then leave the rack sitting in the ethanol for 1 minute
S. Repeat in two more 100% ethanol changes
T. Dip slide rack into xylene 10 times then leave the rack sitting in the xylene for 3 minutes
U. Repeat in 2 more xylene changes

Preparation for Processing Tissue

A. Prior to submission to TACMASR

a. Note whether samples are in 10% buffered formalin or 70% ethanol. Samples should be placed in 10% buffered formalin for up to 24 hours after they have been collected. Formalin penetrates tissue a 1mm per hour. After that time samples should be transferred to 70% ethanol to be held for processing.

B. TACMASR

a. TACMASR encourages investigators to fix samples in formalin for 24 hours and transfer them to 70% ethanol before bringing them to TACMASR.
b. For any samples brought to the lab in formalin, TACMASR staff should transfer them to 70% ethanol and note on the container 70% ethanol as well as the date and time transferred on the investigators TACMASR worksheet.
c. Dispose of the spent formalin in the chemical waste container located behind the fume hood.
d. Samples are routinely processed every Thursday afternoon.
e. Prepare cassette labels using the Brady Labeler and CodeSoft software associated with the computer on the desk space in 0917.
f. When the cassette label has been attached and riveted, place a blue absorbent pad on the work space, using forceps, take the sample out of the investigator vial and transfer it into the labeled cassette. If the sample is small, place it between two cassette sponges or wrap it in blue paper. Sandwiching between two sponges assures the sample remains fixed during processing and does not become lost.
g. Once the sample is in place, snap the cassette lid in place. The cassette lid should fit snuggly up against the cassette. It is important to make sure that no part of the sponge is preventing the lid from closing. That would cause the cassette to come apart during processing, resulting in loss of the sample.
h. Once the sample is in the cassette, the lid firmly attached, place the cassette into a beaker of 70% ethanol.
i.

Processing Tissue

A. Place metal basket containing fixed tissue into processing retort
B. Select appropriate tissue processing program
C. Begin
    a. 70% ethanol fills and drains the retort for 15 minutes while the processor increases and decreases the pressure in the retort and maintains the temperature at ambient
    b. 80% ethanol fills and drains the retort for 30 minutes while increasing and decreasing the pressure in the retort at 40ºC then drains completely from the retort
    c. 95% ethanol fills and drains the retort for 90 minutes while increasing and decreasing the pressure in the retort at 40ºC then drains completely from the retort
    d. 100% ethanol fills and drains the retort for 45 minutes while increasing and decreasing the pressure in the retort at 40ºC then drains completely from the retort
    e. A difference 100% ethanol source fills and drains the retort for 45 minutes while increasing and decreasing the pressure in the retort at 40ºC then drains completely from the retort
    f. A difference 100% ethanol source fills and drains the retort for 45 minutes while increasing and decreasing the pressure in the retort at 40ºC then drains completely from the retort
    g. Xylene fills the retort for 45 minutes while increasing and decreasing the pressure in the retort at 40ºC then drains completely from the retort
    h. A difference xylene source fills the retort for 45 minutes while increasing and decreasing the pressure in the retort at 40ºC then drains completely from the retort
    i. A difference xylene source fills the retort for 45 minutes while increasing and decreasing the pressure in the retort at 40ºC then drains completely from the retort
    j. Paraffin fills the retort for 45 minutes while increasing and decreasing the pressure of the retort at 60ºC then drains the retort
    k. A second paraffin source fills the retort for 60 minutes while increasing and decreasing the pressure of the retort at 60ºC then drains the retort
    l. A third paraffin source fills the retort for 60 minutes while increasing and decreasing the pressure of the retort at 60ºC then drains the retort
    m. The tissue is removed from the basket and placed into a warm embedder for next steps

Sectioning tissue

A. Preparations
    a. Warm a water bath to 45ºC and remove dust particles with kim-wipe
    b. Place tissue blocks to be sectioned onto an ice block topped off with water near the edge of the container
B. Sectioning
    a. Place cooled tissue block onto microtome's chuck
    b. Angle the chuck to meet the edge of the blade parallelly
    c. Turn microtome wheel to close the space between the tissue block to the edge the blade
        i.
    d. Cut into block to revel tissue under the paraffin. Adjust the angle of the chuck as needed moving slowly to not cut into tissue and have tissue loss
    e. Collect tissue ribbon and tranfer to heated bath
    f. Allow for the tissue to expand in the water for 30 seconds
    g. Introduce slide to heated water bath at 45º
    h. Tap on tissue section and drive it towards the slide
    i. When paraffin has met the slide, pull the slide out of the water slowly upwards perpendicularly with the water
    j. Place the slide upward to allow it to dry overnight.
    k. Clean water bath with kim-wipe

Immunofluorescence organoids (8 well chambered slide)

1. Remove media from each well.
2. Fix organoids by adding 200uL/well, prewarmed 3.7% para-formaldehyde at room temperature for 20 mins.
3. Wash with 200uL/well DPBS (Fisher Scientific, 21-040-CV), 5 min, RT
4. Proceed to staining or wrap with parafilm and store at 4^0C
5. Gently remove PBS
6. Permeabilize with 0.5% Triton X-100 in PBS (PBST) for 20min, RT 200uL/well.
7. Wash with 0.01% PBST, 5 min, RT 200uL/well.
8. Block with 2% donkey serum (Jackson Immuno Research, # 017-000-121) in 0.01% PBST 1hr, RT 200uL/well.
9. Remove blocking and add Primary antibody, diluted in 0.01% PBST overnight at 4^{o}C 200uL/well.
10. Wash the organoids with 0.01% PBST, 5 min RT.
11. Add secondary antibody (dilute antibody in 0.01% PBST) and Hoechst (dilute 1:1000 in 0.01% PBST) RT, 1hr dark
12. Wash the organoids with 0.01% PBST, 5 min RT 200uL/well.
13. Store in PBS (500uL/well), 4^{o}C, wrap with parafilm and aluminum foil until confocal imaging.

[all antibodies must be diluted following the product sheet. Also use the same host as primary antibody. eg., if primary is anti-rabbit, use secondary donkey anti-rabbit]

IMMUNOSTAINING

A. Fixation

a. Place the fresh tissue within two blue pads and put the pads within cassettes.
b. Incubate the cassettes in 4% formaldehyde solution, overnight in room temperature.
c. Wash the cassettes containing tissue section in DPBS.
d. Store the tissues in 70% Ethanol at 4^0C until ready to do paraffin embedding and sectioning.

B. Immunohistochemistry

Deparaffinize and Hydrate Tissue

a. Xylene for 3 minutes. Repeat 3X (xylene can be reused from previous staining).
b. 100% ethanol for 1 minute. Repeat 3X.
c. 95% ethanol for 1 minute
d. 75% ethanol for 1 minute
e. Wash in MQH_2O for 5 minutes. Repeat 2X (NB: do not allow tissue to dry at any stage)

Antigen Retrieval

f. Dilute Antigen Unmasking Solution (Vector Labs) to 1: 100 with water. A polyproylene jar (10 slides) holds approximately 500ul, use 50ml of solution in 49.5ml of water.
g. Submerge the slides in the boiling solution for 10 minutes
h. Let the solution cool at room temperature for 20 minutes and transfer slides into PBST (PBS + 0.01% Triton-X 100) wash, 5 minutes
i. Repeat wash 2 times

Blocking Endogenous Peroxidase Activity

J. Incubate tissue for 30 minutes in the dark with 0.3%H_2O_2/Methanol (2.5ml of 30% H_2O_2 in 250ml Methanol)
k. Equilibrate in PBS/0.01 % Triton X-100 for 20 minutes
l. Incubate tissue in 20% goat serum made with PBS/Triton X-100 for 20 minutes In other immunostains the blocking serum used should be the serum of the animal in which the secondary antibody was developed).

Immunostaining

m. Tap off excess blocking serum
n. Add primary antibody (in this case mouse anti-BrdU, 1:40 dilution made in PBS/TritonX-100)
o. Incubate for 2 hours at room temperature
p. Rinse in PBS/Triton X-100 for 5 minutes. Repeat 2X

q. Incubate tissue with 1 :200 dilution of goat anti-mouse biotinylated IgG (NB: anti-mouse-IgG is used since BrdU antibody is an anti-mouse) for 30 minutes. At the same time make ABC reagent (according to the manufacturer's instructions, Vector Laboratories) and allow to incubate at room temperature for at least 30 minutes.
r. Wash in PBS/Triton X-100 for 5 minutes. Repeat 2X
s. Incubate tissue with ABC reagent for 30 minutes.
t. Wash in PBS/Triton X-100 for 5 minutes. Repeat 2X
u. Make DAB solution (following the manufacturer's instructions, Vector Laboratories) and, incubate with tissue for 5 minutes.
v. Rinse slides in MQH_2O 2X
w. Counterstain with Hematoxylin (Gill's No.1) for 45 seconds. **(NB: Hematoxylin will stain darker the longer it is left on the slide)**
x. Rinse well in MQH_2O

Dehydration

y. 75% ethanol for 1 minute
z. 95% ethanol for 1 minute
aa. 100% ethanol for 1 minute. Repeat 3X. Xylene for 3 minutes. Repeat 3X.
bb. Cover slip slides using Permount

C. Immunofluorescence

Deparaffinize and Hydrate Tissue

a. Xylene for 3 minutes. Repeat 3X (xylene can be reused from previous staining).
b. 100% ethanol for 1 minute. Repeat 3X.
c. 95% ethanol for 1 minute
d. 75% ethanol for 1 minute
e. Wash in MQH20 for 5 minutes. Repeat 2X (NB: do not allow tissue to dry at any stage)

Antigen Retrieval

f. Dilute Antigen Unmasking Solution (Vector Labs) to 1 :100 with water. A polyproylene jar (10 slides) holds a proximately 500ul, use 50ml of solution in 49.5ml of water.
g. Submerge the slides in the boiling solution for 10 minutes -
h. Let the solution cool at room temperature for 20 minutes and transfer slides into PBS wash, 5 minutes.
i. Repeat wash 2 times.

Blocking and incubating with primary antibody

j. Incubate tissue in 20% goat serum made with PBS/0.01 % Triton X-100 for 20 minutes
(NB: In other immunostains the blocking serum used should be the serum of the animal in which the secondary antibody was developed).
k. After 20 minutes tap off excess 20% goat serum and incubate with primary antibody either ,4^0C overnight in a humidified chamber or 1-2 hours room temperature (the conditions and antibody concentration will depend on the antibody you are using).

Secondary antibody

l. After primary antibody incubation wash slides with PBS/0.01 % Triton X-100 2 times, 5 minutes each wash. Stain slides with secondary antibody that is fluorescently conjugated IgG for 1 hour at room temperature.
m. After secondary antibody incubation wash slides with PBS/0.01 % Triton X-100 2 times, 5 minutes each wash.
n. Cover-slip slides using aqueous mount with DAPI. Note that when doing confocal you must use Antifade mounting media with DAPI and 1.0 coverslips.
o. View slides under fluorescent microscope or confocal.