Molecular Cytology Core Facility - Efforts to Accelerate the Research Process

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SERVICES

Sample Preparation for *in situ* Molecular Detection

Manual and Automated Experiments
(IHC, IF, RNA *in situ* Hybridization, Histological Stains)

Optical Microscopy and Image Analysis
Histology Services

Dissection and fixation of tissues:
Appropriate dissection and fixation of tissues is very important to achieve satisfactory results in all histological procedures.
Our facility would be happy to share all the expertise and knowledge to provide you with the best reagents and training for dissection and fixation of your tissue samples.

Embedding and Sectioning:
The MCCF trains researchers to process their tissue samples for paraffin embedding and freezing.
We offer two paraffin embedding stations and paraffin ovens for manual embedding.
Most steps of the protocol for paraffin embedding have been automated thanks to our tissue processor.
We train and assist researchers to section their samples and prepare slides with tissue sections for various in situ molecular detection and imaging techniques.

Histological staining:
We carry more than 30 different dyes used in histological staining and have established protocols for more than 30 different methods of histological staining.
Protocols and reagents are also available for some enzymehistochemistry methods, such as x-gal and alkaline phosphatase staining.

![Histological Staining Image]

- Hematoxylin & Eosin
- Crystal Violet
- Cresyl violet Acetate
- Periodic Acid Schiff (PAS)
- Nile Blue + Sudan Black
- Methyl Green
- Fontana-Masson for pigments
- Acid Fast Stain
- Papanicolaou (PAP) Staining
- Safranin O
- Toluidine Blue O
- Acidine Orange
- Methylene Blue
- Evan's Blue
- Luxol fast blue-Modified Kluver's for myelin sheath
- Eosin Y
- Hematoxylin
- Standard Giemsa
- Prussian Blue (Iron) Staining
- Nuclear Fast Red
- Fast Green FCF
- Light Green
- Feulgen Staining
- Oil Red O
- Masson's Trichrome Staining
- Grocott's Methenamine Silver Method
- Benzidine staining for Red Blood Cells
- Alcan Blue
- Alizarin red for cartilage and bone

In situ molecular detection:
Various techniques to detect molecules like proteins and nucleic acids in their natural environment have been practiced with great success at our facility.
Many researchers have been trained to master these techniques by us as well.
We have experience working with very diverse samples, ranging from paraffin and frozen tissue sections to different types of whole mount preparations, like early post-implantation embryos, seminiferous tubules, spheroids, embryonic bodies and others.

In situ techniques performed manually:
- Immunohistochemistry (IHC)
- TUNEL for detecting apoptosis
- RNA in situ hybridization with DIG or radiolabeled RNA probes
- Whole mount IHC/IF staining of early post-implantation mouse embryos
- Whole mount IF staining of oocytes and seminiferous tubules
- Whole mount fluorescence (Yo-pro-1) staining of mouse embryos for apoptosis
- Staining for F-actin with Phalloidin on sections and whole mount preparations
- Other specialized staining techniques on sections or whole mount preparations

Sample preparation for microscopy:
- Mounting and cleaning of whole mount samples for confocal microscopy
- Preparing of adherent cells on chamber slides for live imaging
- Preparing of cells in suspension for live imaging
- Fluorescence counterstaining and mounting of various types of samples for imaging
Examples of Histological Stains

- Silver staining of Aspergillus spores
- Oil red staining of adipocytes
- Trichrome staining of skin
- Alcian blue staining of embryo
- PAS staining of testis
- X-Gal staining of testis
ABOUT MCCF AUTOMATED HISTOLOGY

The MCCF has four automated machines that can run 100 slides per day. The facility offers 124 established protocols, in addition to 152 protocols developed at the facility for our users.

Molecular Cytology Core Facility carries the following services for molecular in situ detection:
- cell proliferation
- stem cell potential & differentiation
- cell signaling
- cell death and senescence
- angiogenesis
- lymphangiogenesis
- hypoxia

MCCF ANTIBODIES

**Established Protocols:** We carry experiments with antibodies we provide. We also constantly carry experiments with antibodies, provided by the users. Records about the new antibodies we validate are strictly kept. Thus, we are able to advise the researchers, whose projects may require some of the antibodies, about the source of the proper reagent. This assures good quality of the results.

**For Testing New Antibodies:** If the protocol is not established, and a new antibody is tested, at least eight slides with identical tissues are required to test various dilutions and conditions. A copy of the antibody specification sheet along with the lot number is needed.

**Antibodies Available at the MCCF (validated on mouse tissue)**
- BrdU
- β-Gal
- β-Tubulin IV
- Calnexin
- CD34
- CD45
- Cre
- Chk1
- C-Kit
- Cyclin D1
- DAZL
- Desmin
- Disabled 2/96
- ERG
- Estrogen Receptor
- EMA-1
- FAK
- Fragilis
- Fibronectin
- GFAP
- GFR
- Glucagon
- GM130
- Insulin
- LC3
- Ly61
- Laminin
- Mab414
- Meaco-32
- MMP9
- Nanog
- Nestin
- Neurofilament
- P21
- Parp Cleaved
- PCNA
- P-Chk1
- P-MAPK
- PLZF
- P-S6 Ribosomal Protein
- Olig-2
- Podoplanin
- Prox-1
- P-Stat3
- PTEN
- RhoB
- Shh
- Survivin
- Tcr-119
- VASA
- VWF

**Antibodies Available at the MCCF (mouse and human tissue cross-reactive)**
- Androgen Receptor
- β-Catenin
- Carbonic Anhydrase IX
- CD3
- Cl.Caspase 3
- E-Cadherin
- Giantin
- Glut-1
- Iba1
- Ki-67
- N-Cadherin
- Ox-Phos
- P27
- P53
- p-Akt
- Parkeratin
- p-Histone H2AX
- p-Mek 1/2
- Snail
- Sox2
- SMA
- Vimentin

**Antibodies Available at the MCCF (human tissue – specific)**
- CD4
- CD8
- CD31
- CD68
- CD105/Endoglin
- FoxP3
- Ki-67
- Vimentin

Methods:
- Immunohistochemistry [IHC] (single/double)
- Immunofluorescence [IF] (single/double/triple)
- Non Radioactive RNA in situ hybridization RNA ISH]
- Double Non Radioactive RNA in situ hybridization and immunohistochemistry [RNA ISH-IHC]
WIDEFIELD MICROSCOPES

Widefield Microscopes
Widefield Microscopes are equipped with bright field, dark field, fluorescence, phase contrast and DIC imaging capabilities.

- 2 Zeiss Axioplan 2 Imaging upright microscopes (ZRC)
- 1 Zeiss Axiocam 200M “Metamorph” inverted microscope with motorized stage for imaging cultured cells (ZRC)
- 2 stereomicroscopes (Zeiss Lumar V.12 in RRL and Leica MZFLIII in ZRC) for imaging large whole-mount tissues or organisms

Zeiss Mirax Slide Scanner
Zeiss Mirax Slide Scanner automatically digitizes your slides for easy viewing and storage. Digitized images can be zoomed up to 40x magnification or more with high quality and can directly be captured or analyzed. The time it takes to scan each slide depends on the size of the section and can vary from 5 to 15 min.

CONFOCAL MICROSCOPES

Confocal Microscopy
Confocal Microscopes are capable of high magnification optical sectioning for visualization and localization of fluorescent signals at cellular level.

- 2 Leica TCS SP2 (one upright and one inverted) capable of 1- or 2-photon laser scanning (ZRC)
- 1 Zeiss LSM510 laser scanning confocal microscope (RRL)

Live Imaging
Live Imaging of cells and embryos ex vivo can be performed on a number of our microscopes that are equipped with environmental chambers.

- Zeiss LSM 510 Live line scanning confocal microscope is capable of taking very fast confocal images of live samples in order to study fast dynamic processes in cells. (RRL)
- Perkin Elmer spinning disk confocal microscope also has high acquisition rate with minimized bleaching. (ZRC)
- Zeiss Axiovert 200M “Metamorph” widefield microscope is recommended for high resolution phase/DIC and fluorescent images of live samples. (ZRC)

Image Processing & Analysis
Image Processing and Analyses are a crucial part of digital microscopy. We have multiple licensed image analysis software to meet all of your quantification needs. We will assist and train you to conduct the following image analyses:

- 3D reconstruction and 3D rendering
- Maximal intensity projection
- Colocalization
- Automated counting of cells
- Fluorescence intensity measurements
- Measurements of lengths and branching points of blood vessels
- Morphological measurements

Metamorph Image Analysis Software
Intravital Microscopy

Chronic window preparation

**Rabbit ear chamber**
Transparent window chamber implanted in the ear of the rabbit
Monitoring the angiogenesis during wound healing

**Dorsal skinfold chamber**
First used by Algire (1940) for similar studies
Tolerated well, may last around 30-40 days

**Cranial window preparation:**
To study brain tumors
Lasts up to 1 year
Tumor Vessels

Dorsal skin chamber

Tumor cells (cell tracker red)

Vessles (FITC- dextran)

Multiphoton scan
Seminiferous Tubule

- Unique functional unit of somatic and germ cells
- Site of mitotic divisions of both somatic and immature germ cells
- Site of meiotic division of germ cells
- Contains spermatogonial stem cells and also multipotent germline stem cells
- Accepts/sends signals through testis interstitium
- Site of specialized ectoplasmic specifications controlling precisely germ cell differentiation, maturation and release of spermatozoa
OUR APPROACH- EXAMPLES

Cell Proliferation Markers
BrdU, P-Histone H3, Ki-67, PCNA, P-Histone H2AX, Survivin

Spermatogonial Differentiation Markers
PLZF, c-Kit, Cyclin D1, DAZL, Vasa, Id2, E-Cadherin

Cell Signaling
PMAPK, PMEK1/2, P S6 Ribosomal Protein, FAK, β- Catenin

Intercellular Junctions
N-Cadherin, E-Cadherin, Zo-1, β- Catenin
P-Histone H2AX Immunohistochemistry

First frame
P-Histone H2AX Immunohistochemistry
Epifluorescence and Virtual Images of Testis Sections

PLZF (green), Vasa (red)  DAZL (green), Vasa (red)  PLZF(green), DAZL (red)
Data Analysis
β– Catenin and Vimentin Protein Expression

(Double immunofluorescence: β- Catenin (green), Vimentin (red), DAPI (blue))
PLZF (green) and FAK (red)
Three Molecular Markers: PLZF (yellow), FAK (green) ZO-1 (red) and DAPI (blue)
Spermatogonia and Intercellular Junctions

PLZF (yellow)
β- Catenin (red)
ZO-1 (green)
DAPI (blue)
Functional Domains

PLZF (red)
P S6 Ribosomal Protein (green)
DAPI (blue)
Actin Cytoskeleton and Spermatogonia

PLZF (green)
Phalloidin (red)
DAPI (blue)
CONCLUSIONS

• Sample Preparation is Very Important Even for Optical Imaging.
• Our Approach is Applicable to Wide Variety of Samples.
• Simultaneous Detection of Several Markers \textit{in situ} Helps in Understanding Functional Relationships.
Analysis of SSH mRNA by *in situ* Hybridization
Mirax scan of radioactive SSH mRNA in situ
Quantification using Metamorph

- Draw an AOI
- Set color threshold
- Log region statistics
DISCERNING VIABILITY OF EXCISED LIVE LIVER TISSUES

- Determining the viability of cells in assessing the effectiveness of various clinical interventions.

- Mitochondrial activity and membrane integrity are the two parameters often used to discriminate live and dead/dying cells.

- We evaluate two fluorescent live cell dyes, a live mitochondrial marker MitoTracker Red (MTred), and an apoptosis marker Yo-Pro-1, in differentiating live versus apoptotic cells in whole-mount liver tissues.
Apoptotic marker Yo-Pro-1 is present in most liver cells despite the presence of MTred and absence of Tunel staining.
In Normal Mouse Liver Samples Yo-Pro-1 Enters All Cells

In Other Normal Mouse Tissues Yo-Pro-1 and MTred Signals are Mutually Exclusive
Intact Lobe of Mouse Liver

Yo-Pro-1 Staining was Exclusive to a Subset of Cells and was Confirmed by Tunel Staining

Apoptotic Cells are Detected by Tunel Following 30 min Incubation of Liver Tissue
Level of MT red Staining is not Uniform in Intact Mouse Liver Lobe and Intestinal Tissue
Our hypothesis
Physical dissection of the liver triggers a signaling cascade that opens channels or pores in the plasma membrane, allowing Yo-Pro-1 to rapidly permeate the cell and stain the nucleus.

Future studies are required to explain the liver-specific mechanisms.

Conclusions

• Abundant YO-PRO-1 staining in the liver shows that plasma membrane integrity is very easily compromised.
• This phenomenon is common across species (human, mouse and pig), but not across different organs.
• YO-PRO-1 staining is ubiquitous even in liver tissues that were not ablated.
• Physical damage of the liver organ upon dissection triggers apoptosis.
• YO-PRO-1 is not an appropriate dye to assess whether cells in the liver have survived the RFA treatment.
• Metabolic activity level of mitochondria can differ greatly from hepatocyte to hepatocyte. It may be a reflection of functional dynamic.

References
Sofocleous et al. (2008) Radiology 249:364
Boffa et al. (2005) Transplantation 79(7):842
Tunneling Nanotubes Provide Routes for Intercellular Communications

- Long non-adherent F-actin-based cytoplasmic extensions connecting proximal or distant cells.

- Sensitive to physical stimuli, light and mechanical stress, resistant to trypsin.

- Have diameter ranging from 50 nm to 800 nm.

- Allow continued intercellular communication even when cells move in different directions.
Scratch assay movie
Transmit cytosolic components and cell organelles bidirectionally between cultures of mesothelioma cells (Lou E. et al., in preparation)
Tunneling nanotubes in fresh intact samples of tumors resected from patients with malignant pleural mesothelioma or poorly-differentiated lung adenocarcinoma
Analysis of Circulating Tumor Cells

Overview
About 15 min scanning, using 10X obj, total 300-400 frames / well

Microscope
UltraView
Spinning Disk Confocal
MetaMorph
Epi-fluorescence Microscope

Screening ROI

Detailed imaging
Using 40X or 63X oil
AR_Ai 647
Pankeratin
CD45_Ai 488
DAPI

Report
Diagnose
Image analysis
Immunofluorescence chamber slide scanning

Pelkin Elmer Ultraview Spinning disk confocal microscope

Tile scan with 10X objective

Detailed imaging using 40x objective, overlay
Z stack movie 1
Patient Sample: CD45 (green) and pankeratin (red)
Ki-67 Immunofluorescence- Thick Tumor Section