Fluorescence Microscopy

BIOE202 Spring 2016

Dr. Karin Jensen
Questions from last lab/lecture?
Clarification: Medical uses of tissue staining

• H&E stain
  – Hematoxylin-blue stain that is BASIC/positive (binds acidic/negatively charged components, ex. DNA/RNA)
  – Eosin-red stain that is acidic/negative (binds acidophilic (i.e. Basic)/positive, ex. Most cytoplasmic proteins)
• Primary diagnostic technique for pathologists
• 2.5-3 million slides per day!
THANK YOU for your feedback!

Assignments have increased my understanding of course concepts?
Never 1—2—3—4—5 Always
THANK YOU for your feedback!

I have a solid understanding of the Critical Thinking Questions listed at the end of the lab handouts.

Nope 1—2—3—4—5 Absolutely
THANK YOU for your feedback!

The pace of the course is...
Too Fast 1—2—3—4—5
THANK YOU for your feedback!

How well prepared do you feel for technical writing assignments?
Not Well 1—2—3—4—5 Very Well
THANK YOU for your feedback!

How well do you understand the concept of aseptic technique?
Not Well 1—2—3—4—5 Very Well

![Bar chart showing the number of students' ratings.]

- Number of Students
- Rating

- Rating 1: 5 students
- Rating 2: 10 students
- Rating 3: 0 students
- Rating 4: 5 students
- Rating 5: 20 students
THANK YOU for your feedback!

The lectures are interesting.

Nope 1—2—3—4—5 Absolutely

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Number of Students

Rating

0  5  10  15

1  2  3  4  5
THANK YOU for your feedback!

The lab quizzes help me keep up with course material

Nope 1—2—3—4—5 Absolutely

![Bar graph showing the number of students rating the quizzes. The ratings range from 1 to 5, with the following distribution: 1 rating has 2 students, 2 rating has 1 student, 3 rating has 5 students, 4 rating has 10 students, and 5 rating has 15 students.](attachment:bar_graph.png)
THANK YOU for your feedback!

I feel comfortable plotting in Matlab.

Nope 1—2—3—4—5 Absolutely
THANK YOU for your feedback!

What is the best part of the class so far?

- Labs, Lectures, office hours
- Historical perspective/real-world uses
- Low stress environment in labs
- TAs and lab assistants
- Friendly/approachable course staff
- Availability of course staff
- Reviewing assignments soon after they are due
- Candy 😊
THANK YOU for your feedback!

What is the least useful part of the class so far?

• Plotting in Matlab
• Quizzes - memorization, stress
  – How can we help reduce quiz stress?
• Lecture
  – In addition to data review, what would you prefer to see covered in lecture?
• Experiment/Purpose before lab
THANK YOU for your feedback!

What would you like to see change in the course and why?

• More discussion of lab purposes/steps in protocol
  – More description will be added to protocols and more discussion in class

• Guidelines for writing
  – Rubric has been posted on the course website, please let me know if you have questions!

• Coming in on Saturdays
  – Unfortunately this is unavoidable due to the type of work we are doing, we have minimized this by asking the TAs/lab assistants to cover some of these tasks, for example, changing differentiation media for 2 weeks

• Review data in class (upcoming slide)

• Consistency in TA grading
  – TAs grade on a rubric and discuss assignments together before assigning grades
THANK YOU for your feedback!

What would you like to see change in the course and why?

• Time for meeting with TAs for plotting and ImageJ
  – If you need more help please email Dr. Jensen or a TA-we are more than happy to meet with you!

• Assignments to be due on Fridays
  – All deadlines will be moved to Fridays

• More than 2 credits
  – This is a tough one

• Videos
  – This is a great idea! We are looking into this!

• Website-live links/shorter link names
  – I’m working on this!
THANK YOU for your feedback!

Based on your feedback these changes will be implemented:
Data review/discussions in class
Quizzes—focus on more overview/purpose type questions, quiz replacement policy already addressed
More in-class discussion of assignments when they are posted
Critical Thinking Questions: creation of a discussion board on Compass? Other ideas?
THANK YOU for your feedback!

Final items:
Problems with lab groups—please talk to Dr. Jensen and/or your TA

Are there topics we should cover?

If you did not get to submit feedback last week, please do so this week.

Feedback is welcome at any time!
What exactly is Fluorescence?

• Green Fluorescent protein (GFP)-Module 2

White light

UV light

GloFish pet
What exactly is Fluorescence?

- Green Fluorescent protein (GFP)-Module 2

** Principle of Fluorescence **

1) A photon hits a type of molecule called a fluorophore.
2) By absorbing the photon, the fluorophore (electron) enters a higher energy state.
3) Some of the energy is lost due to thermal fluctuation.
4) When the fluorophore returns to its ground state, it releases energy in the form of a photon. Note that the energy of the emitted photon is always **lower** than the absorbed, so it has a **longer wavelength** (Stoke’s shift).

Photos **promote** an electron from a fluorophore to a **higher energy level**.

When it returns to the **ground state** they will emit a **lower energy photon**.

[Principle of Fluorescence Diagram]

Why Use Fluorescence for Microscopy?

- Sensitivity
- Selectivity

https://www.microscopyu.com/galleries/fluorescence/cells.html
Fluorescent Microscopy

• What you can expect to see
  – Black background with green fibers visible (Alexa fluor 488 phalloidin-F-actin)
  – Black background with blue circles visible (DAPI-DNA)
  – Keep sample protected from light after stains are added
    • If left exposed too long to light, the fluorescence will fade. This is called quenching or photobleaching

Merged image
Review: Fixing cells

Fixation is a critical step before cell staining

Why?

• Preserve cells and tissue structure
• Prevent autolysis (self digest by enzymes)
• Prevent putrefaction (decomposition)
Review: How do we fix cells?

• Organic solvents (alcohols or acetone)
  – Remove lipids
  – Dehydrate tissue
  – Denature and precipitate proteins

• Chemical crosslinkers (formaldehyde, glutaraldehyde)
  – Covalent chemical bonds are formed between proteins and surroundings

Formaldehyde
Permeabilizing cells: Triton X-100

- Triton X-100 is a detergent use to permeabilize cell membranes
- Used at 0.1%
- Allows stains to enter the cell

(Why was this NOT necessary for ORO?)
Fluorescence Microscopy

Principle of operation

1) High-intensity light source or laser provides incident photons

2) Filter selects wavelength(s) to excite the fluorophore

3) Dichroic mirror directs light towards the sample

4) Sample absorbs incident light and emits photons at a longer wavelength

5) Dichroic mirror allows emitted light through to ocular (or other detector)

- prevents extra wavelengths including reflectance of exciting light

- ensures that only the excitation wavelength (range) is used

- if emitted wavelength passed then reflectance of that wavelength would be viewed
Fluorescence Microscopy

Considerations

- Brightfield and phase contrast microscopy use *transmitted* light. Fluorescent microscopy utilizes *reflective* light, meaning both the excitation and emission light paths are directed through the objective.

- Filters and mirrors must be chosen to match the fluorophore of interest.

- The high intensity light source can damage live samples and *photobleach* fluorophores, so take care to minimize unnecessary exposure.
Fluorescence Microscope

Mercury Lamp used for Fluorescence
• Emits light better in UV range
• Requires 20min warm-up to reach stable emission
• Has limited lifetime

Inside orange box is a filter wheel we will adjust for each stain
• Contains dichroic mirror, emission filter, and excitation filter
DAPI

4',6-Diamido-2-Phenylindole
Binds to AT regions of DNA

Audra Storm

http://www.lifetechnologies.com/order/catalog/product/D1306
Phalloidin Alexa Fluor 488

- Phalloidin is a toxin from the deadly *Amanita phalloides* mushroom that binds F-actin

[Image of Phalloidin molecule and spectrum]

[Image of fluorescent stain with green signal]
Online tutorial

- http://media.invitrogen.com.edgesuite.net/tutorials/1Intro/player.html
Other Applications of Fluorescence

- **Flow cytometry**
  - Measure individual cells based on fluorescent staining

![Image of flow cytometry diagram](http://flowcytometry.med.ualberta.ca/wp-content/uploads/2015/04/Flow-Cytometry-how-it-works-Diagram.jpg)

![Image of flow cytometry data](https://wwwbdbiosciencescom/in/research/tcell/sampledata/indexjsp)
Other Applications of Fluorescence

- Fluorescent resonance energy transfer (FRET)
  - Detect molecular interactions

![Diagram of FRET process](https://en.wikipedia.org/wiki/Förster_resonance_energy_transfer)

http://www.nature.com/scitable/content/fret-visualizes-the-ran-gradient-14620325
Several safety concerns this week

- DAPI is a mutagen
- Phalloidin is toxic
- Formalin is toxic
- The SDS for DAPI, phalloidin and formalin are in the ELN

- Lab safety glasses must be worn at all times (except imaging)
Schedule and logistics

• Lab next week:
  – Main Lab: Fluorescence microscopy
  – No Return Lab
  – No lecture next week
  – There WILL be lecture the week of the lab practical

• Lab hours-sign up for extra practice with Dr. Jensen or a TA

• Online sign ups will be sent out tomorrow

• Lab practical week of March 14th
Final Project Ideas

Let us know what you are interested in!