Measuring gene expression and protein production

Module 3, Lecture 5

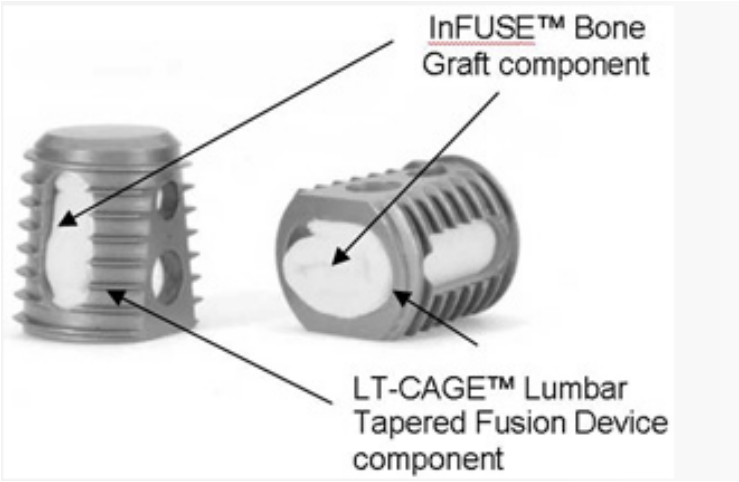
20.109 Spring 2014

From M3D4 lecture

Challenges in orthopedics and beyond

- C. H. Evans, *Tissue Eng B* 17:6 (2011)
- Only three orthopedic technologies with clinical trials!
- Huge publication:product ratio
- Translational research doesn't advance careers (incentives)
- Perfect as the enemy of the good

"At what point is it best to stop tweaking and move forward to the next phase of development?"



OTOH: Medtronic Inc said it agreed to pay **$85 million** to settle… [accused] of making misleading statements concerning Infuse (Reuters)

Other wrap up & review from M3D4

- Possible paths to moving TE forward
  - Targeted support at academic/industry levels by TE societies
  - Better academic/industry communication
  - Standards as enforced by journals or grant providers
  - Targeted support by grant-providing bodies (cf now?)
- Concrete examples of potential standards:
  - Characterizing TE constructs (for the same application) by the same methods across labs, to facilitate efficacy comparisons: e.g., cartilage TE proteoglycans all measured by DMMB
  - For a given method, must document specific analysis choices: e.g., threshold for positive signal as *n* std dev above average
  - Having a base set of characteristics to test, even if not by the same exact assay: e.g., for all TE scaffolds test cell viability, compressive strength, standard gene expression, etc.

Existing ASTM standards for TE

Designation: F2212 – 11

Standard Guide for
Characterization of Type I Collagen as Starting Material for Surgical Implants and Substrates for Tissue Engineered Medical Products (TEMPs)[1]

5.7 *Impurities Profile*—The term impurity relates to the presence of extraneous substances and materials in the collagen. These impurities can be detected by Western blots, ELISAs, GC-MS, and other types of assays. The user is also directed to Guide E1298 for additional information. If there is

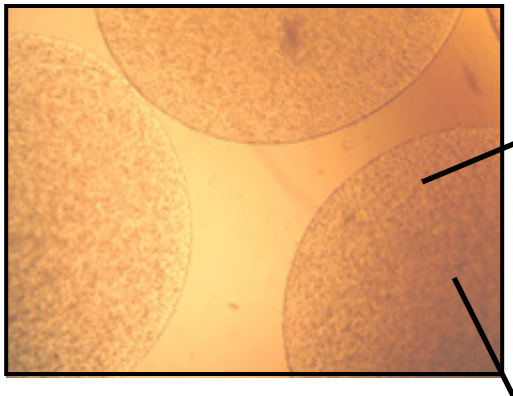
Topics for Lecture 5

- Measuring protein levels
- Measuring transcript levels
- Imaging assays

Sounds boring! Why bother?

- In 20.109, we tell you what assays to perform
  - designs vary, but measurement paths are identical
- In real research, you must decide not only *what* is worth measuring but *how* to measure it
  - sometimes just choosing among existing technologies
  - sometimes inventing something novel or customized
- Hey… this type of thinking also happens to be relevant to the M3 proposal!

Module overview: 2nd half



1. Enzymatic digestions

Test for collagen proteins (by ELISA) and for proteoglycans (with dye)

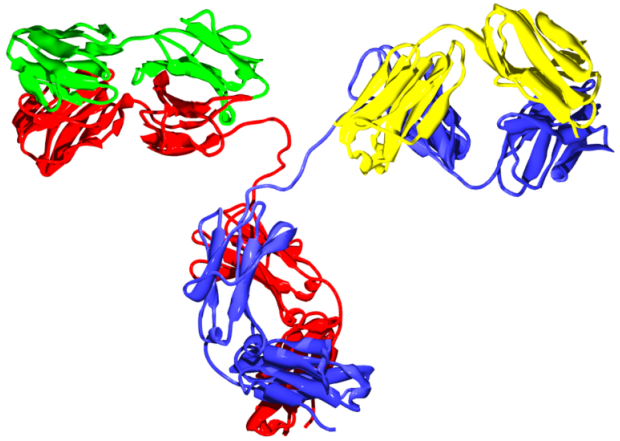
2. EDTA-citrate dissolution

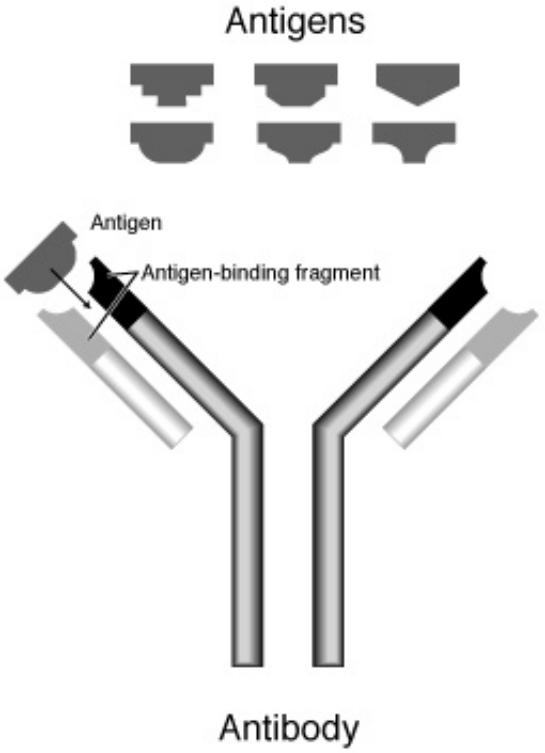
Purify RNA from cells → Prepare complete cDNAs →

Run qPCR to measure CN II, CN I, and 18S rDNA.

Antibodies are specific and diverse

- Specificity
  - variable region binding, K_D ~ nM
  - linear or conformational antigens
- Diversity
  - gene recombination
- Production
  - inject animal with antigen, collect blood
  - hybridomas (B cell + immortal cell)





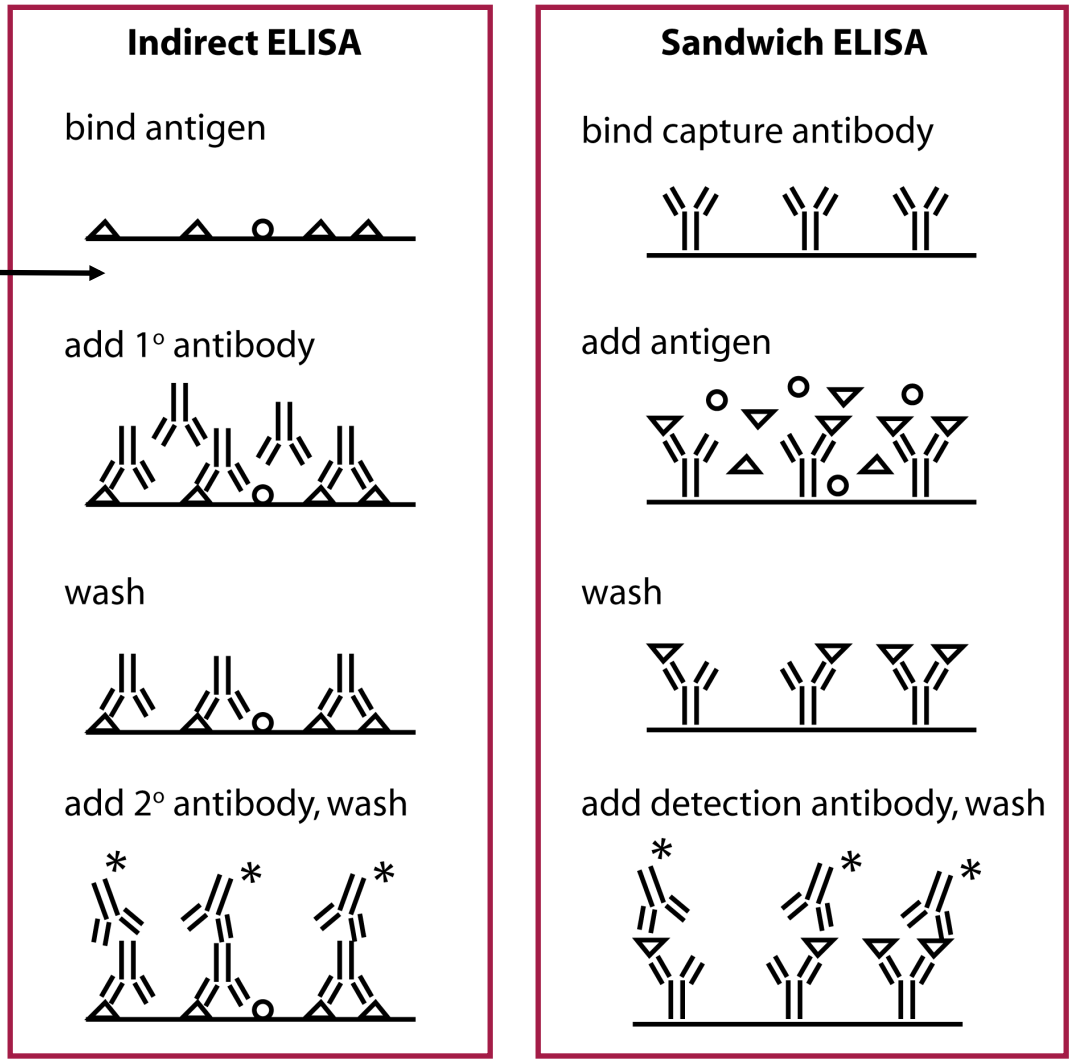
Public domain images
(Wikimedia commons)

Day 5-7: protein analysis by ELISA

- ELISA: enzyme-linked immunosorbent assay
  - specific
  - sensitive
  - multiple kinds

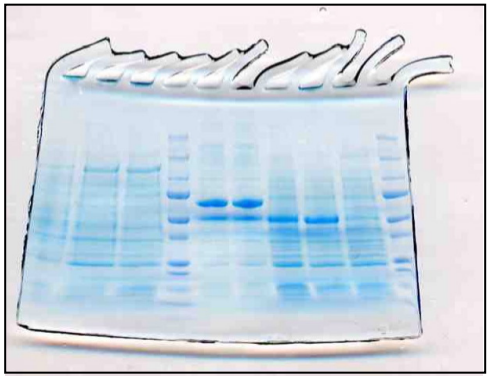
"blocking" step also needed

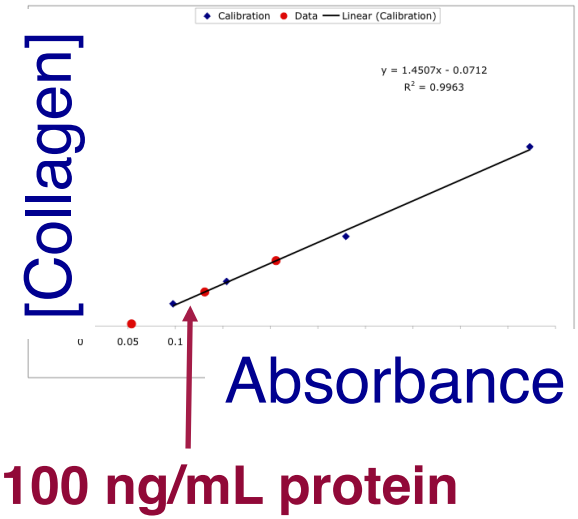
△ = protein of interest



Common protein-level assays

- PAGE
  - simple and low cost
  - Coomassie detection limit ~ 0.3-1 ug/band (2-5 ng/band for silver staining)
  - cannot distinguish two proteins of same MW
- Western blot
  - identifies specific protein
  - detection limit ~1 pg (chemiluminescent)
  - only simple for denatured proteins
- ELISA
  - detects native state proteins
  - quantitative
  - high throughput

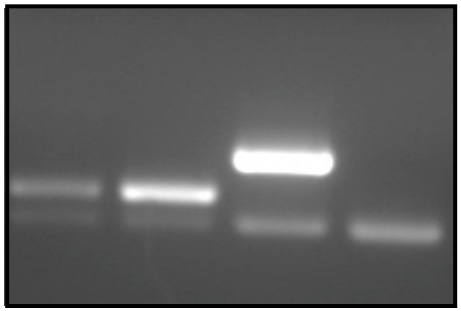




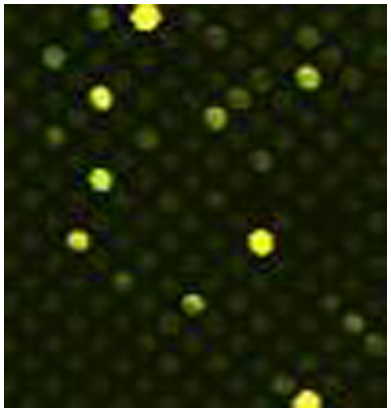
Current Protocols in Cell Biology, Molecular Biology

Common transcript-level assays

- RT-PCR (end-point)
  - simple, low cost
  - can be semi-quantitative
- Microarrays (end-point)
  - spotted c(cDNA)
  - high cost, need specialty equipment
  - complicated and fraught analysis
  - high throughput
- q-PCR (real-time)
  - some special equipment, medium cost
  - highly quantitative
  - multiplexing potential
  - requires optimization (primers)



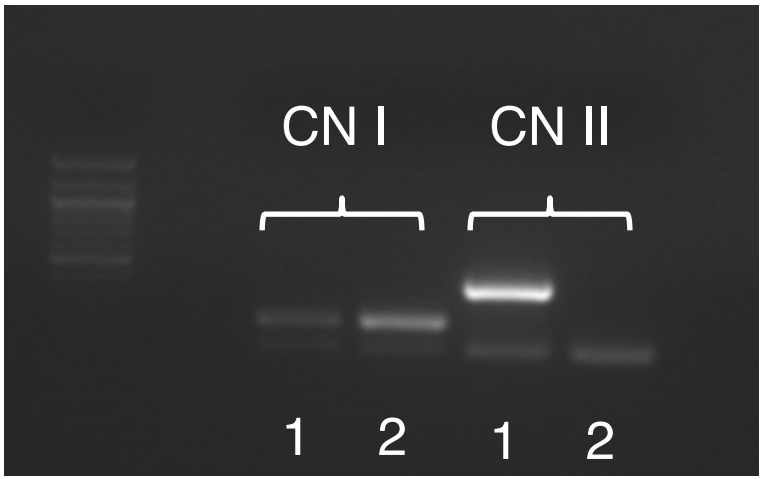
Sample 1: red
Sample 2: grn



Current Protocols in Cell Biology, Molecular Biology

End-point RT-PCR

- Co-amplification in one tube
  - collagen + GAPDH
- Optimize primers
  - no cross-hybridization
  - similar signals (vary [primer])
  - similar efficiency
- Reliability issues
  - must be in exponential phase
  - sensitive to change in [RNA]
- Visualize on a gel
  - measure band intensity/area
  - low dynamic range



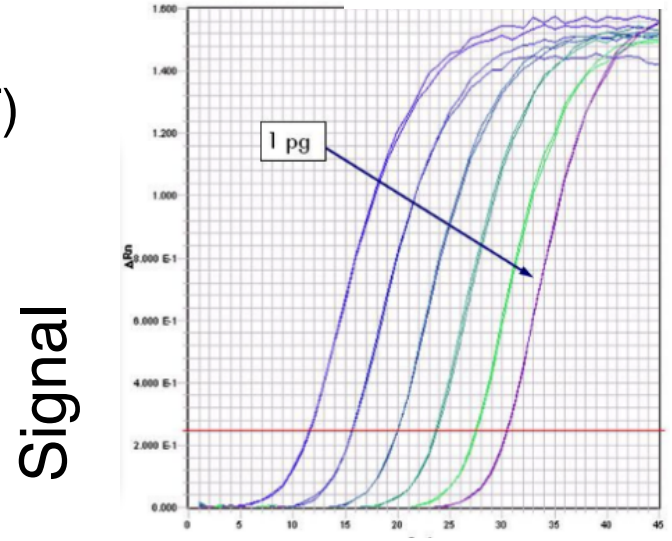
Collagen (upper band)
GAPDH (lower band)

Which sample is from chondrocytes, and which from stem cells?

Introduction to qPCR

- Real-time tracking of [DNA]
- Uses probes that fluoresce
  - when bind to any DNA
  - when bind to specific DNA (FRET)
- How and why does [DNA] change during PCR?
  - first plateau
  - exponential phase
  - linear phase
  - second plateau
  - 1st: detection limit
  - 2nd: competition, reagent limits, inhibition
- Starting point for analysis: threshold cycle C_T

qiagen.com



Interlude: Reproducibility in science

Problem: "In September, Bayer [described] how it had halted [a majority] of its early drug target projects because in-house experiments failed to match claims made in the literature."
http://online.wsj.com Dec 2nd, 2011

See also 2014 Nature (**505**) editorial + proposal by NIH heads.

Solution? "The initiative aims to help scientists validate their research findings by providing a mechanism for blind, independent replication by experts from Science Exchange's network of […] core facilities and contract research orgs."
http://blogs.plos.org/everyone/2012/08/14/plos-one-launches-reproducibility-initiative/

Or just more problems?
http://scholarlykitchen.sspnet.org/2012/08/16/the-reproducibility-initiative-solving-a-problem-or-just-another-attempt-to-draw-on-research-funds/
http://www.xconomy.com/seattle/2012/10/02/the-reproducibility-initiative-a-good-idea-in-theory-that-wont-work-in-practice/

And: reproducibility issues can come from surprising places!

NATURE | NEWS

Male researchers stress out rodents

Rats and mice show increased stress levels when handled by men rather than women, potentially skewing study results.

Alla Katsnelson

28 April 2014

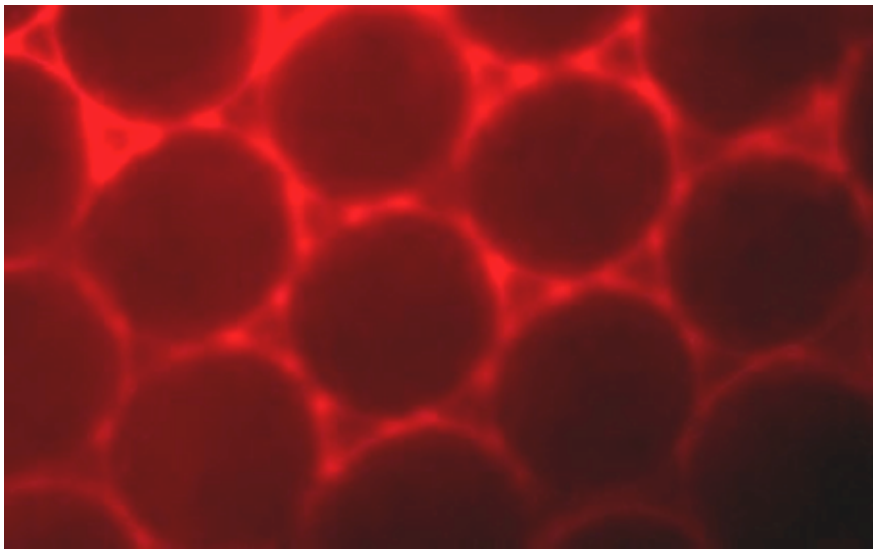
Adam Gault/Getty

Animals handled by men had elevated stress hormone levels.

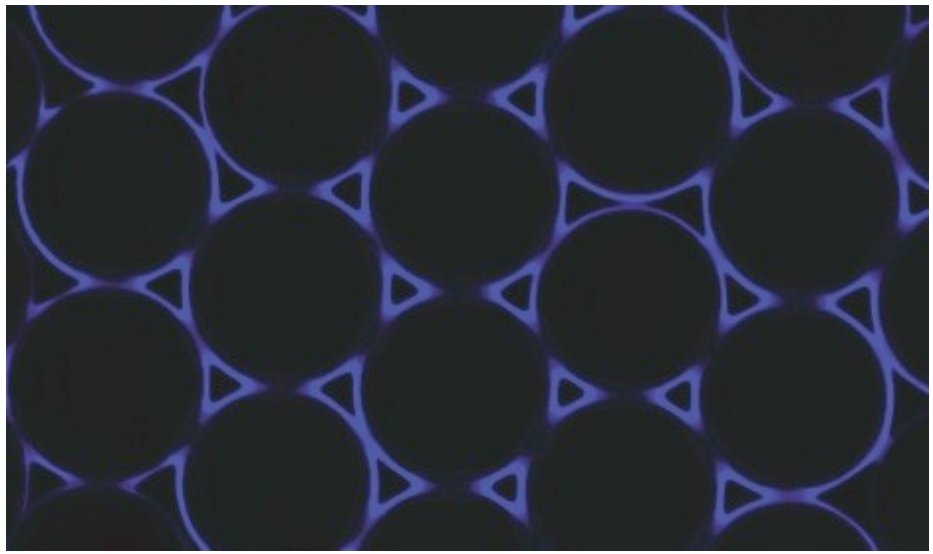
Male, but not female, experimenters induce intense stress in rodents that can dampen pain responses, according to a paper published today in *Nature Methods*[1]. Such reactions affect the rodents' behaviour and potentially confound the results of animal studies, the study suggests.

Image quality in microscopy

- Epifluorescence: noisy due to out-of-plane light
- Confocal: pinhole rids out-of-plane light; scanning

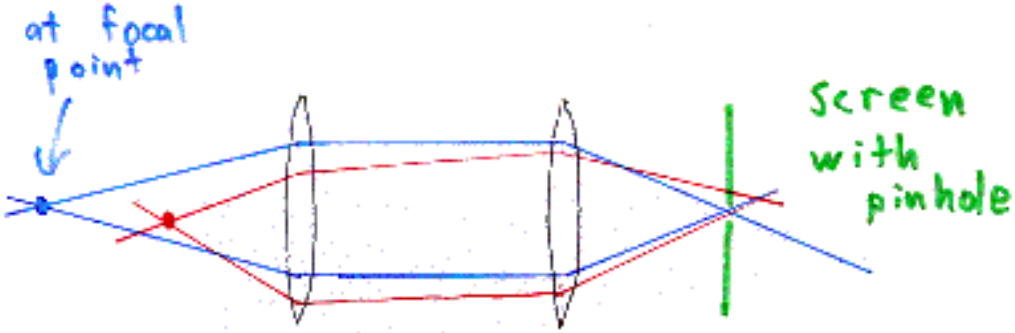


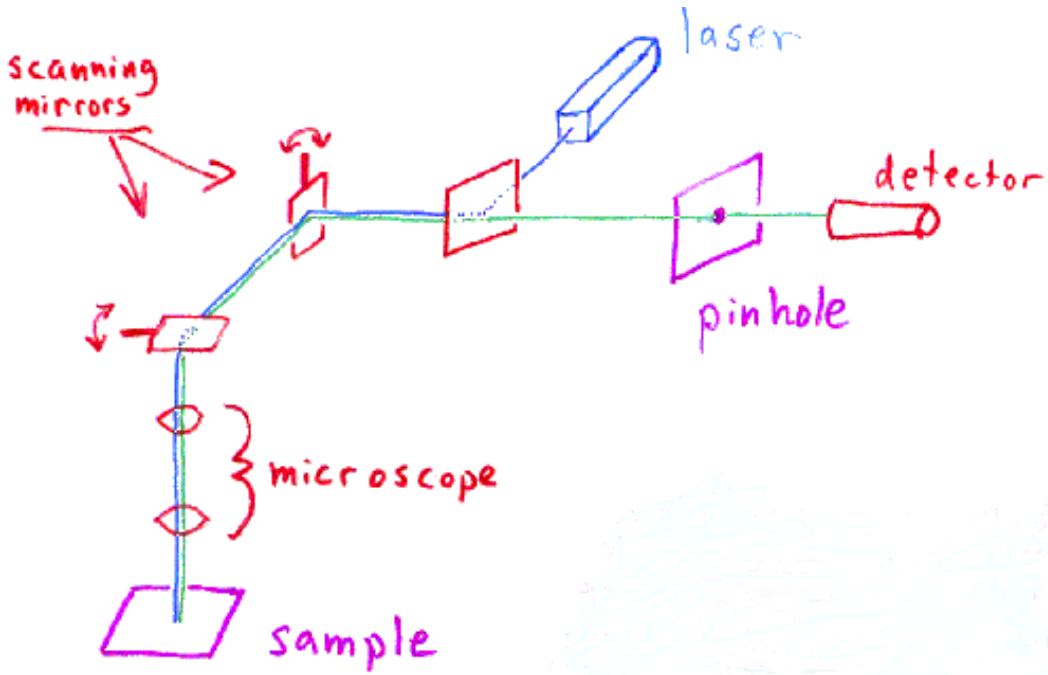
Epifluorescence



Confocal

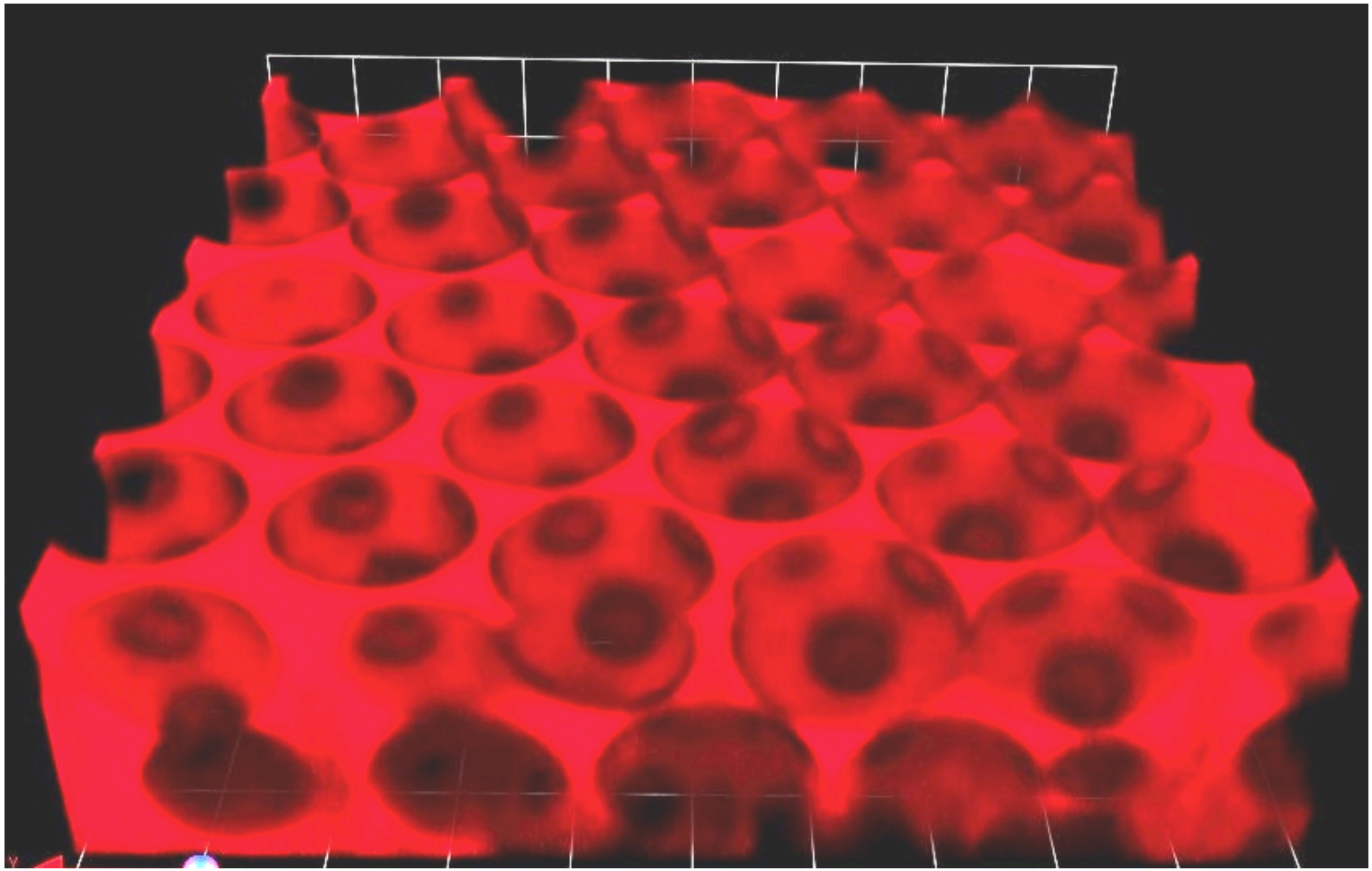
Confocal uscopy: theory



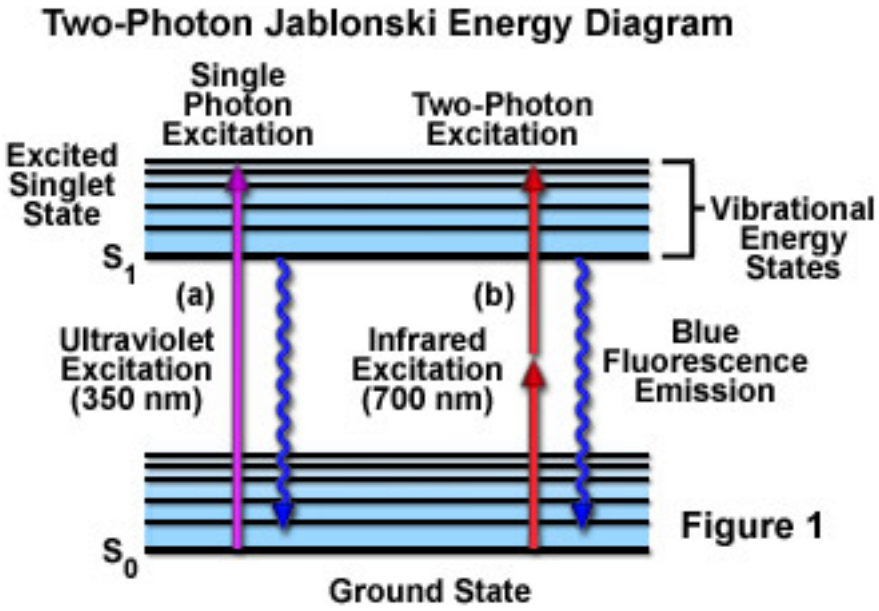


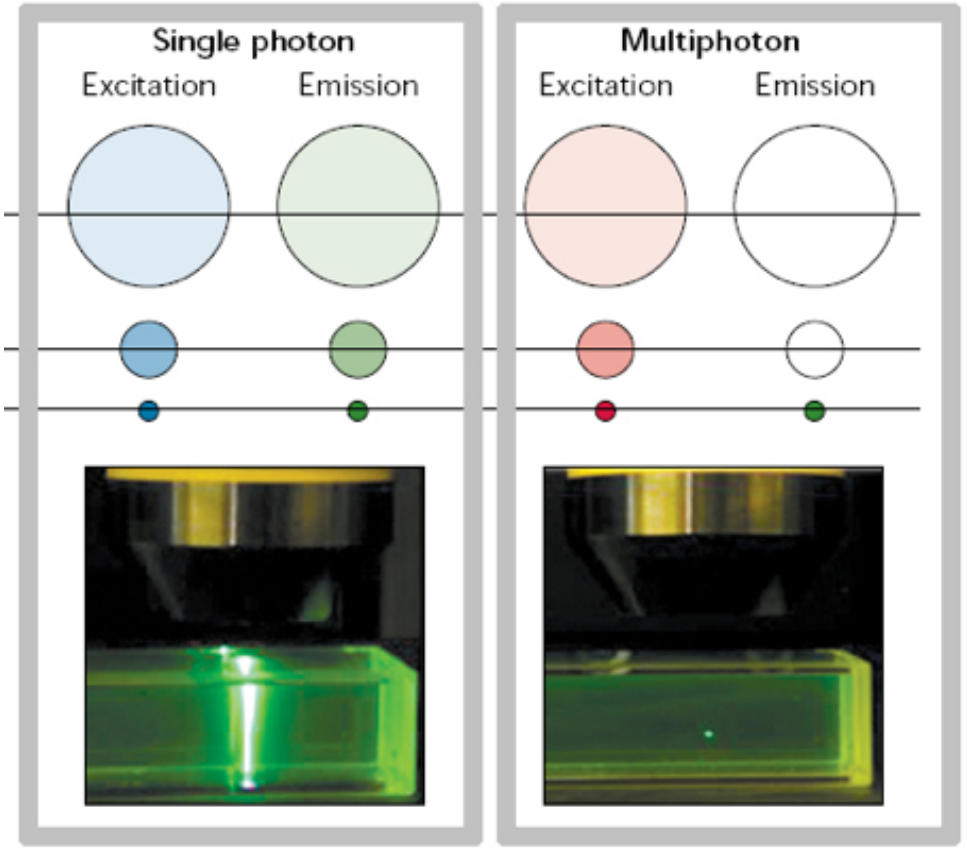
Images from: http://www.physics.emory.edu/~weeks/confocal/

Confocal uscopy permits 3D reconstruction



2-photon microscopy: theory





Images: (1) microscopyu.com; (2) http://parkerlab.bio.uci.edu/microscopy_construction/build_your_own_twophoton_microscope.htm

2-photon microscopy permits deep imaging, even *in vivo*

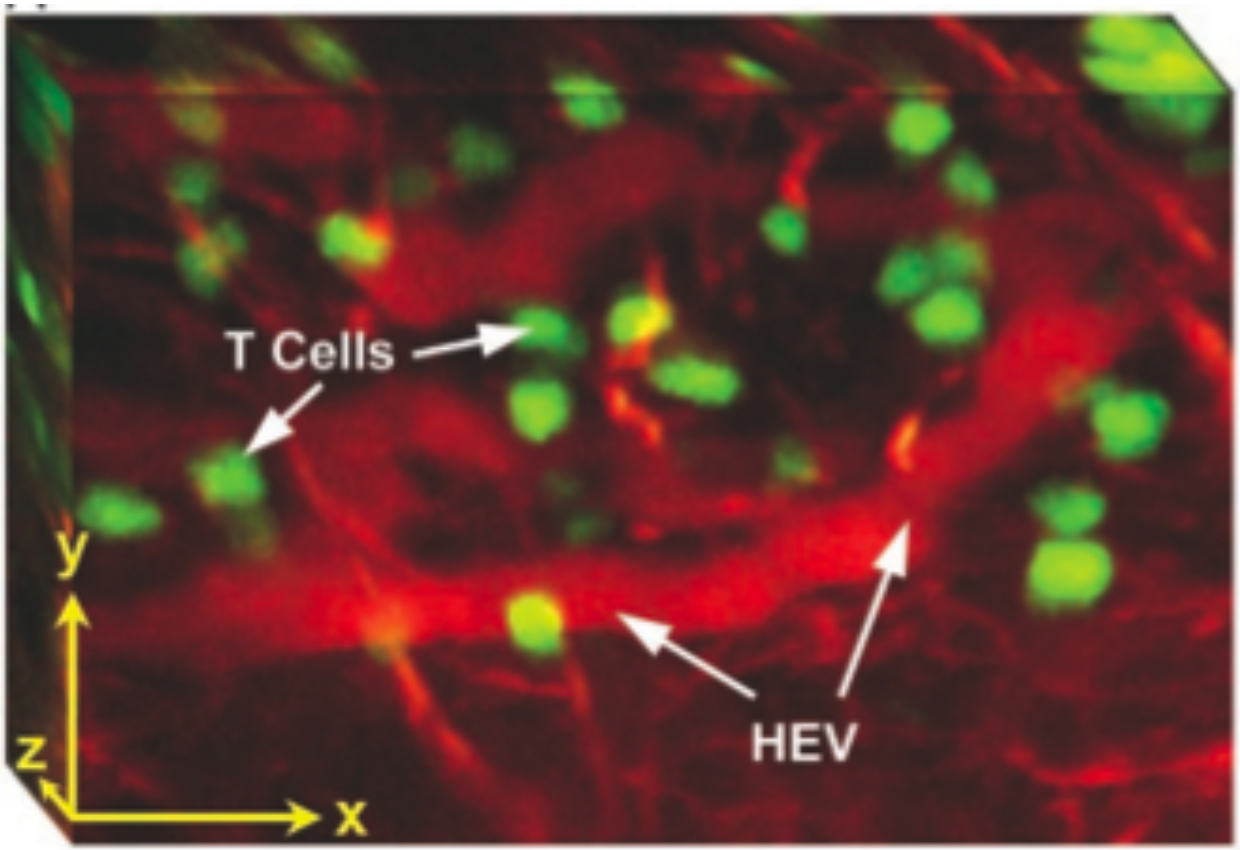


Image from: M.J. Miller, et al. *PNAS* **100**:2604 (2003)

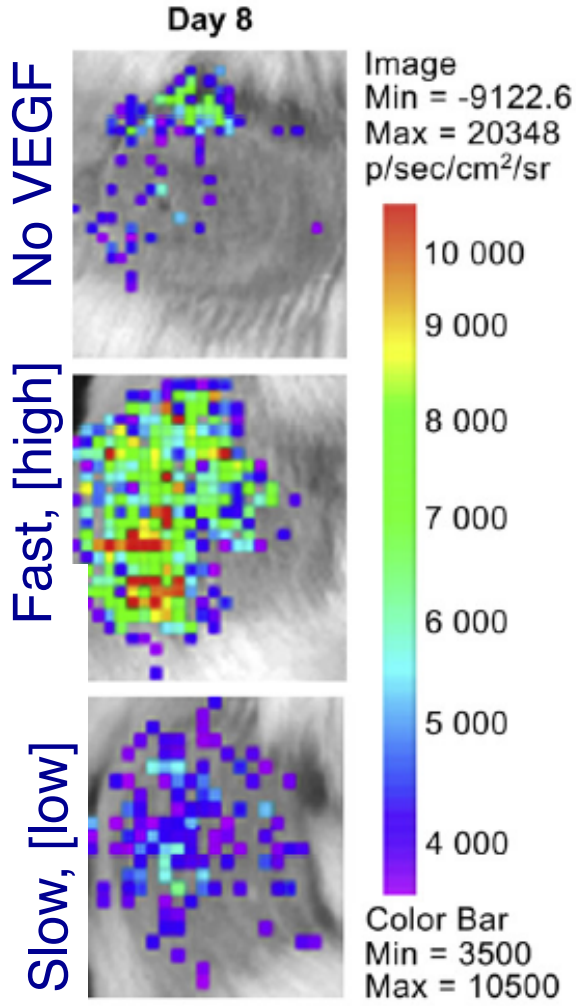
What kinds of information can imaging provide?

- Static measurements
  - overall cell state: viability, apoptosis, signaling
  - specific organelles, cytoskeleton
- Dynamic measurements
  - calcium (or other) fluxes
  - cell motility
- *What is learned from single-cell (uscopy) vs. population (e.g., ELISA) assays? Pros/cons?*

- Different modalities → different information
  - vis-à-vis resolution, depth, coverage, signal:noise

Non-invasive imaging

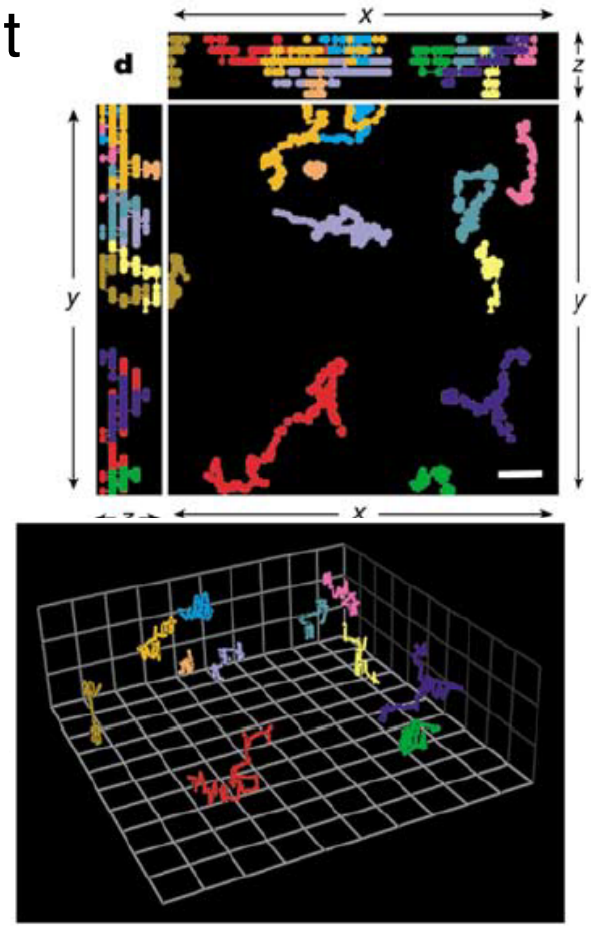
- MRI, tomography, ultrasound, etc.
  - medical diagnostics
  - also measure gene expression
  - fuse gene with reporter
  - whole-body imaging with bioluminescence
- Example: monitoring angiogenesis
  - VEGFR2-*luc* (luciferase reporter)
  - slow- & fast-release VEGF in fibrin scaffolds
  - mice injected with luciferin (substrate) and observed for VEGF receptor upregulation
- Other uses? (think tumors)



M. Ehrbar, et al. *Biomaterials* **29**:1720 (2008)
Nature News Feature **412**:372 (2001)

Day 5-6: image analysis

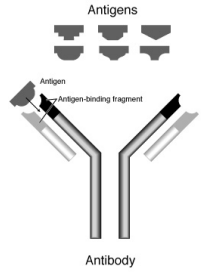
- Imaging data is often high throughput
  - 4D: time, *x-y-z*
  - requires computation, *and*
  - human design/interpretation
- Many available analysis packages
  - some ~ $20-30K
  - NIH ImageJ = free
- Your analyses
  - automated cell counts
  - optional: explore other features

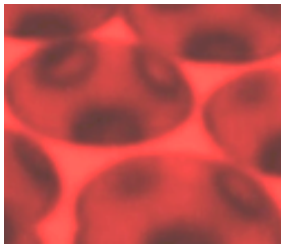


Images from: T.R. Mempel, et al. *Nature* **427**:154 (2004)

Lecture 5: workhorse gene, protein, and imaging assays

- Antibodies to diverse target proteins can be made and used for detection/measurement.
- Trade-offs exist (e.g., between simplicity and accuracy) for different transcript-level assays.
- Fluorescence imaging is a powerful tool for studying cells and materials.





Next time: cartilage TE, from *in vitro* and *in vivo* models to the clinic; qPCR analysis.