Assays for gene expression and protein production

Module 3, Lecture 5

20.109 Spring 2012

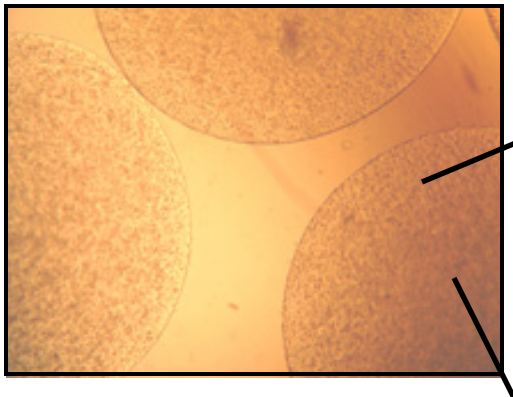
Topics for Lecture 5

- 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)

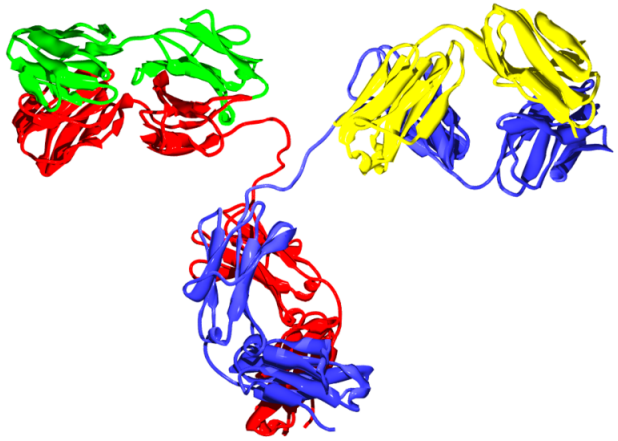
2. EDTA-citrate dissolution

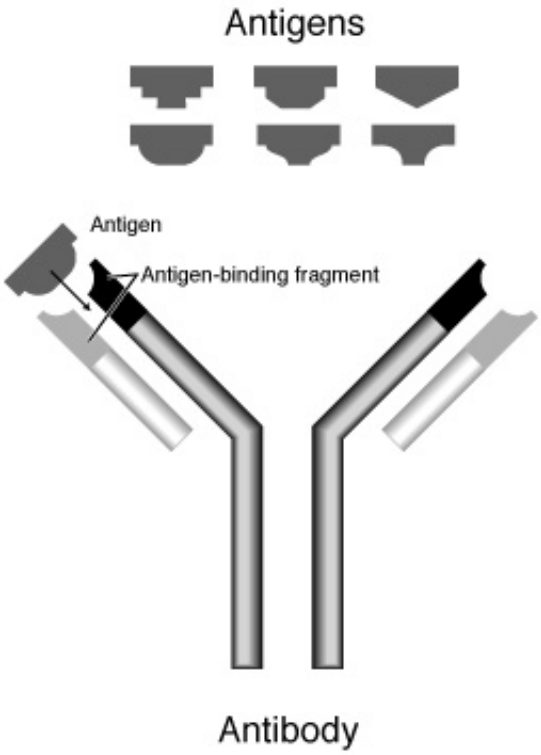
Purify mRNA from cells → Prepare complete cDNAs →

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

Antibodies are specific and diverse

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





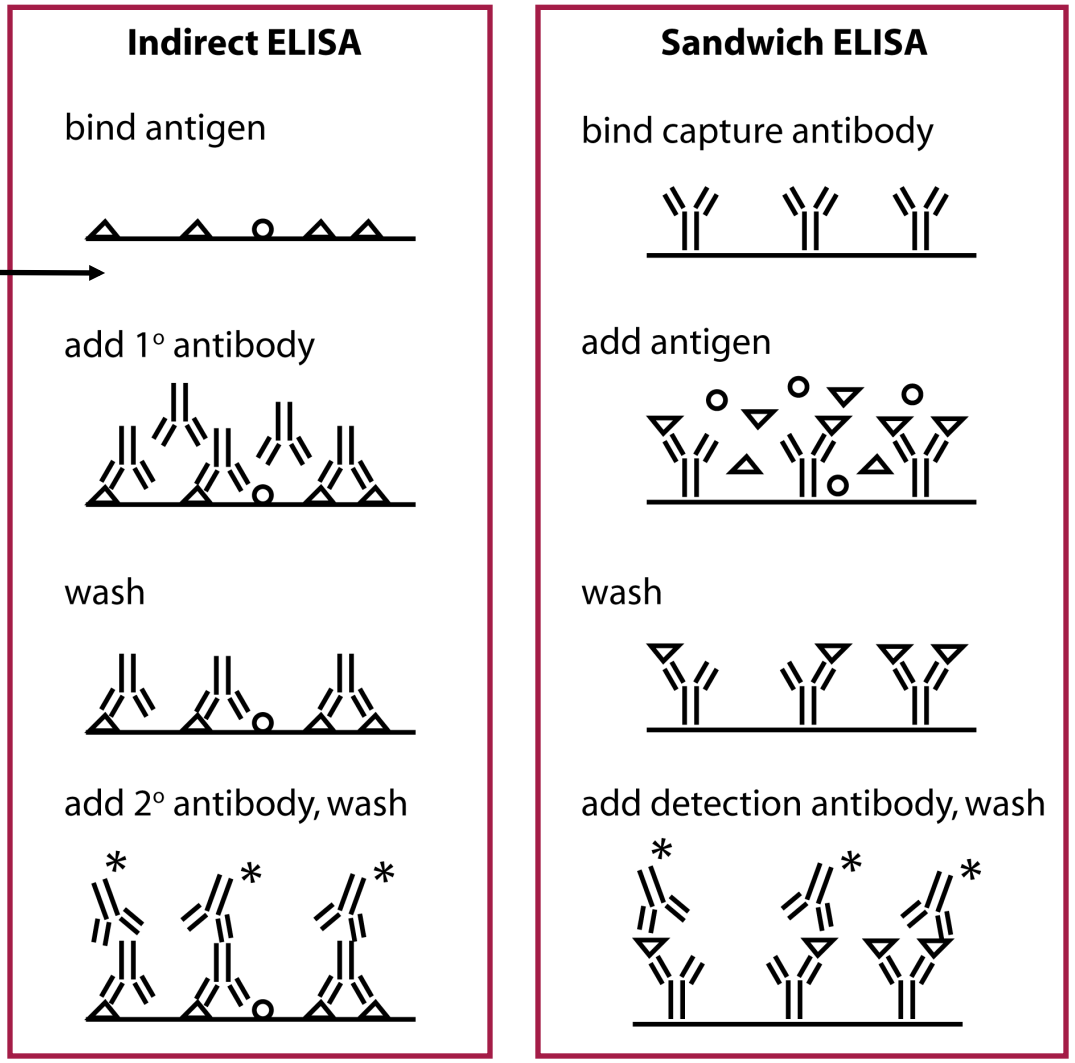
Public domain images
(Wikimedia commons)

Day 5-7: protein analysis by ELISA

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

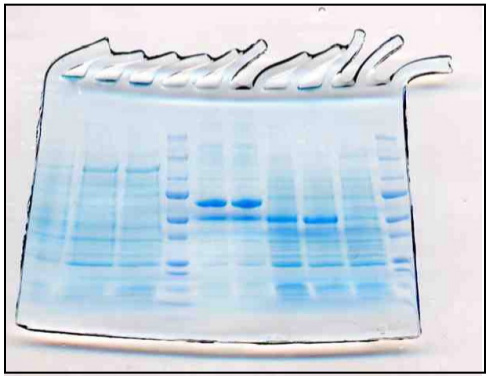
"blocking" step also needed

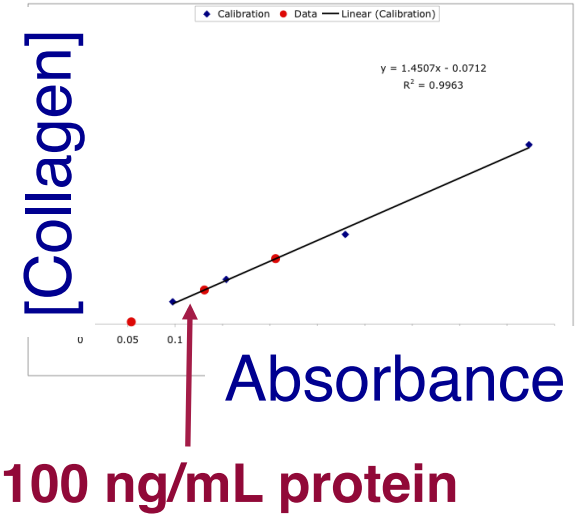
△ = protein of interest



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

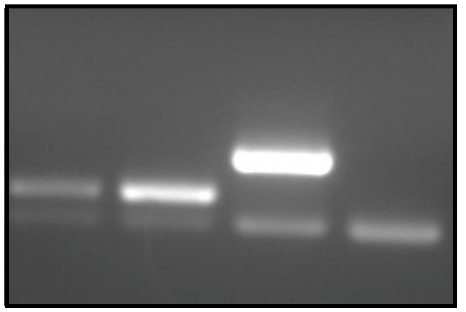


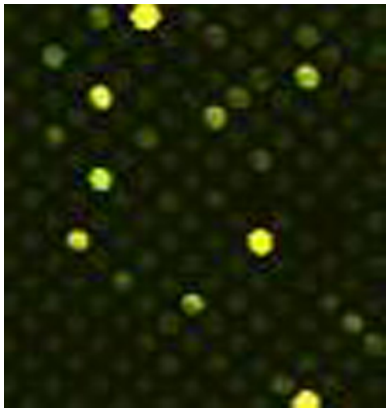


Current Protocols in Cell Biology, Molecular Biology

Common transcript-level assays

- RT-PCR (end-point)
  - simple, low cost
  - can be semi-quantitative
- Microarrays (end-point)
  - 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)

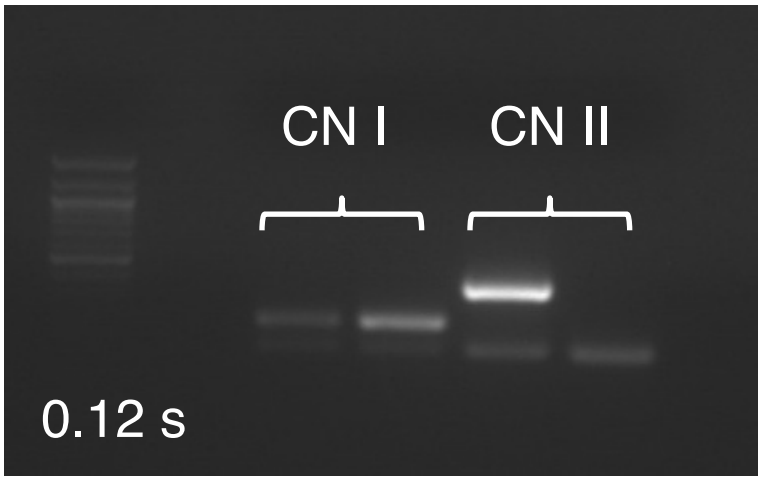




Current Protocols in Cell Biology, Molecular Biology

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



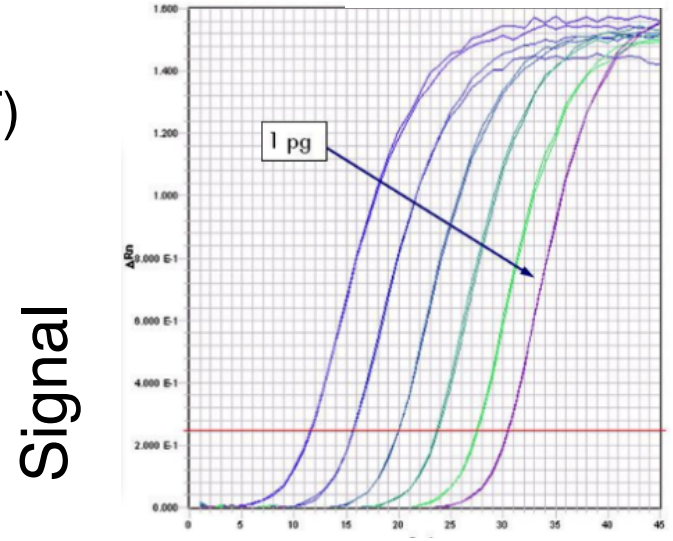
Collagen (upper band)
GAPDH (lower band)

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
  - detection limit
  - competition, reagent limits, inhibition
- Starting point for analysis: threshold cycle C_T

qiagen.com

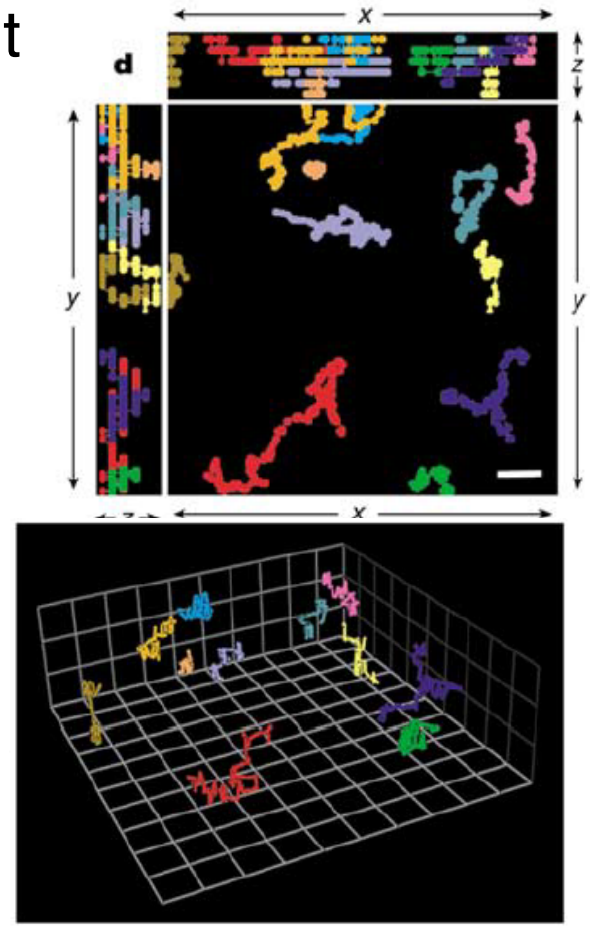


Interlude: science intersecting with politics and the limits of evidence

- Recommendations to reduce *routine* screening for breast and prostate cancers
  - physical/emotional stress of unnecessary biopsies
  - catching cancers that would recede or never spread
  - unlike colon, detected late-stage cancers hardly down, while early stage detection way up
- References
  - S.H. Woolf, *JAMA* (2010) **303**:162 (commentary)
  - “In Shift, Cancer Society Has Concerns on Screenings” nytimes.com Oct 21, 2009

Day 5-6: image analysis

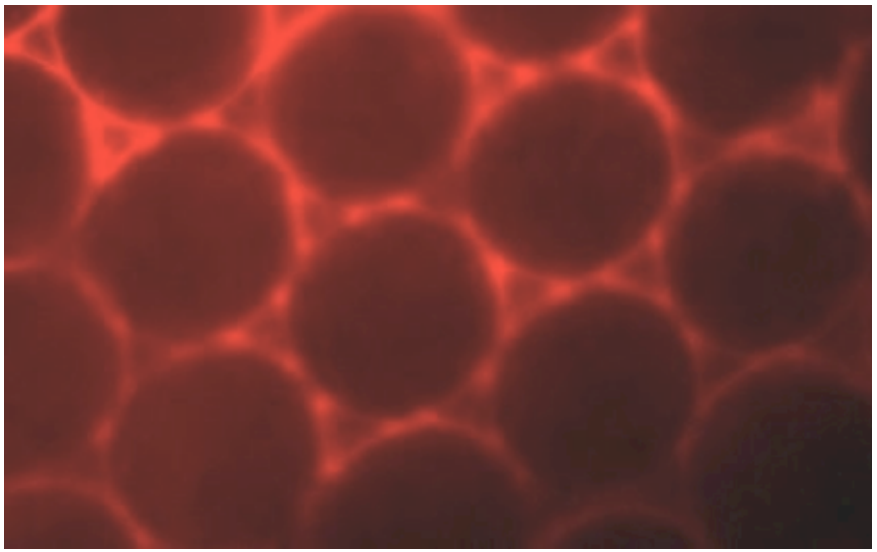
- Imaging data is often high throughput
  - 4D: time, *x-y-z*
  - requires computation, *and*
  - human design/interpretation
- Many available analysis packages
  - some ~ $20-30K
  - NIH ImageJ = free
- Your analyses
  - automated cell counts
  - optional: explore other features



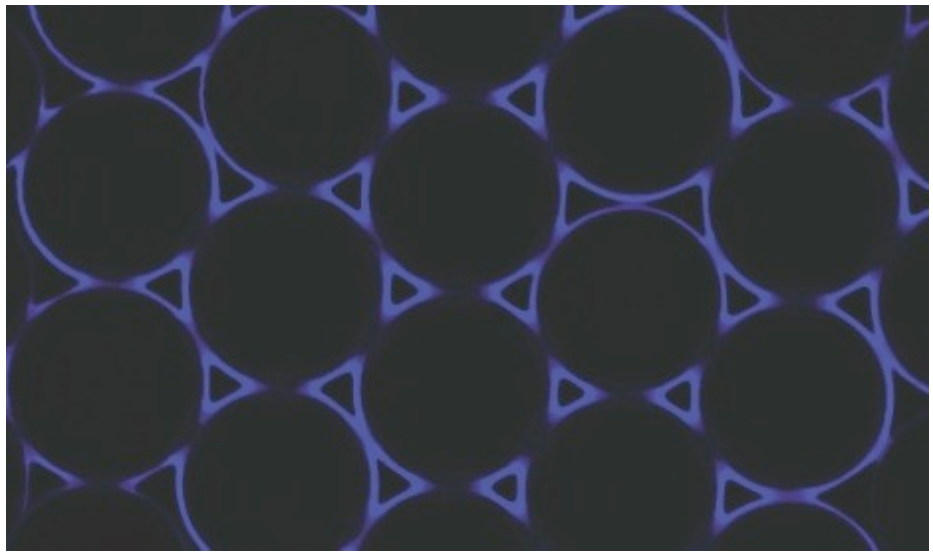
Images from: T.R. Mempel, et al. *Nature* **427**:154 (2004)

Image quality in microscopy

- Epifluorescence: noisy due to out-of-plane light
- Confocal: pinhole rids out-of-plane light; scanning
- 2-photon: femtoliter volume excited; good depth (IR)

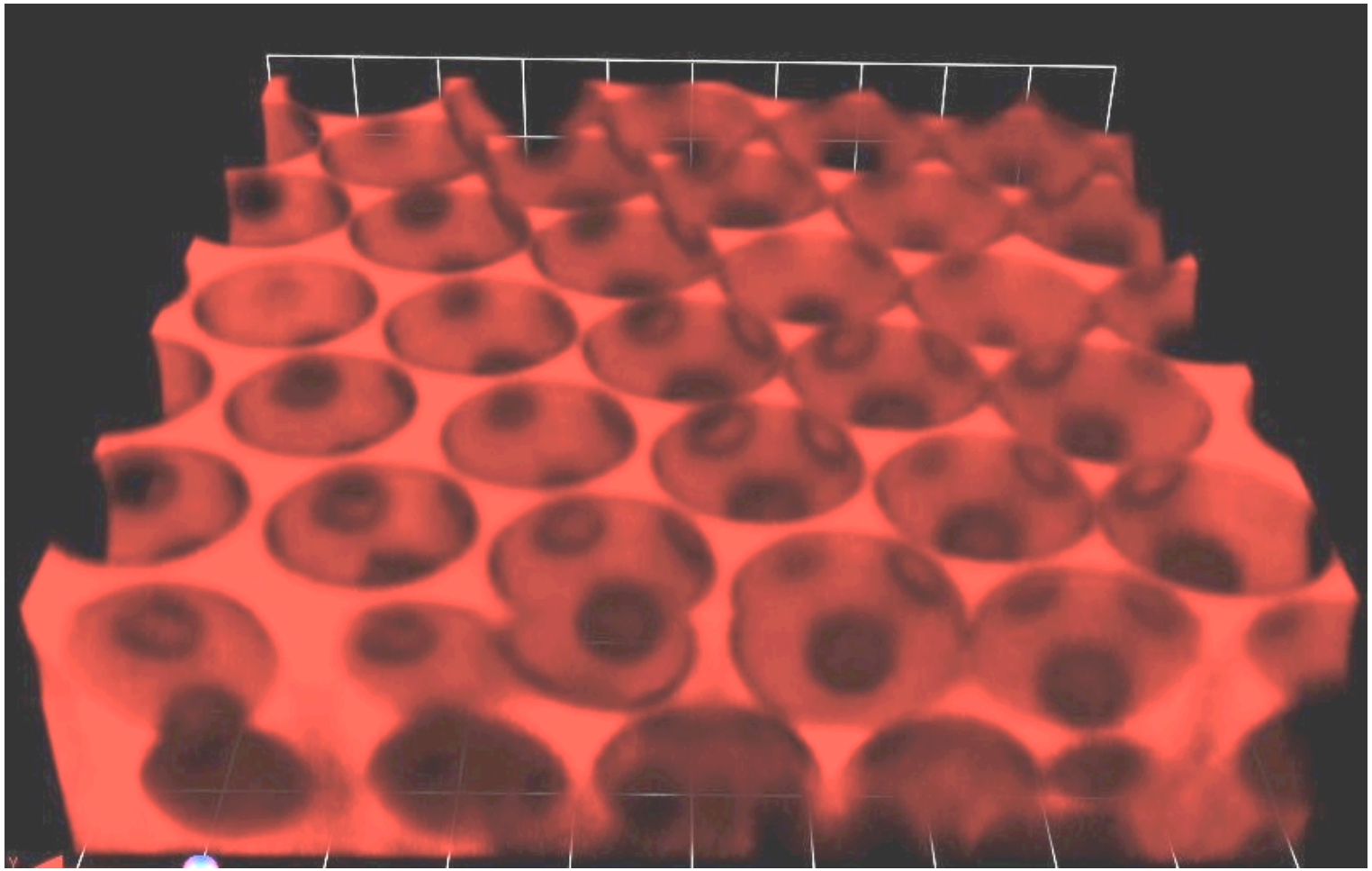


Epifluorescence



Confocal

Confocal uscopy permits 3D reconstruction



Fluorescence microscopy

- Light source
  - Epifluorescence: lamp (Hg, Xe)
  - Confocal: laser (Ar, HeNe)
  - 2-photon: pulsed laser
- Filter cube
  - Excitation
  - Dichroic mirror
  - Emission
  - Band-pass vs. long-pass
- Detection
  - CCD camera: photons → voltages → pixels

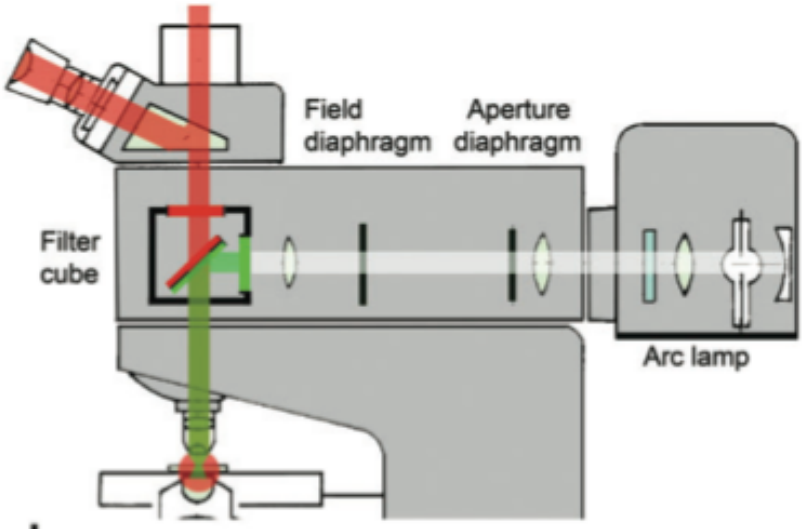
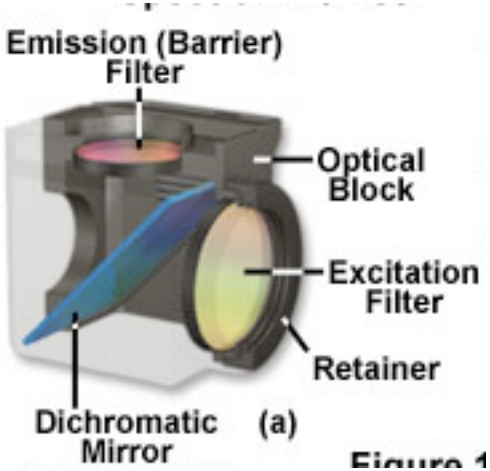
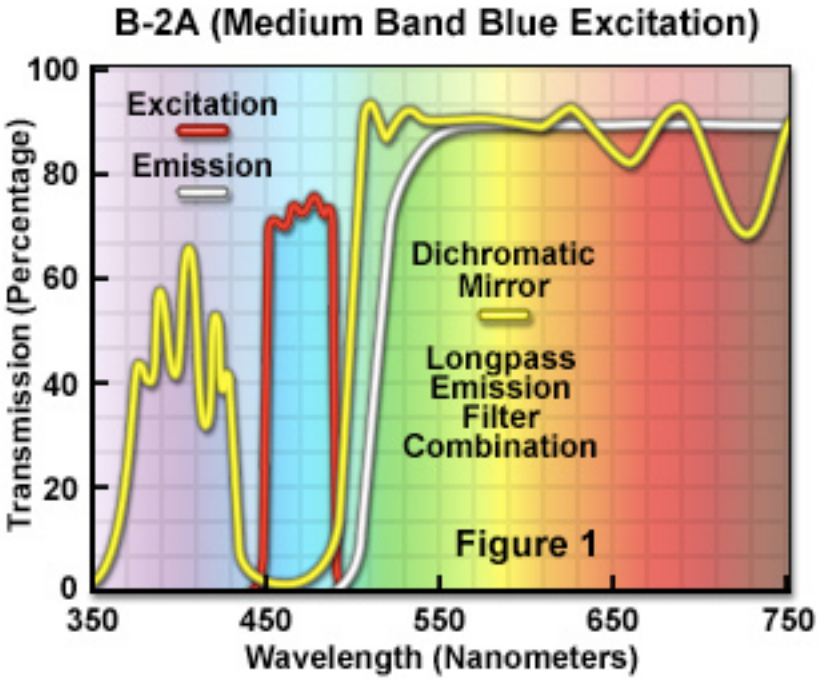


Image from: Lichtman & Conchello, *Nature Methods* **2**:910 (2005)

Specifications for Day 3 imaging

- Live/Dead Dyes
  - Green 490 ex, 520 em
  - Red 490 ex, 620 em
- Excitation 450-490 nm
- Dichroic 500 nm
- Emission 515^{+} nm





Images from: Nikon microscopy website: www.microscopyu.com

Lecture 5: conclusions

- Antibodies to diverse targets (e.g., 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.