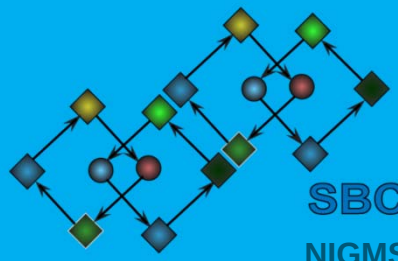

NIGMS funded Center

Experimental Methods in Systems Biology

Part of the Coursera Certificate in Systems Biology

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Department of Pharmacology & Systems Therapeutics
Fall 2014, Week 1, Types of Measurements Part 2
Proteins and Protein States

Icahn School of Medicine at Mount Sinai

Important Features of Any Experiment

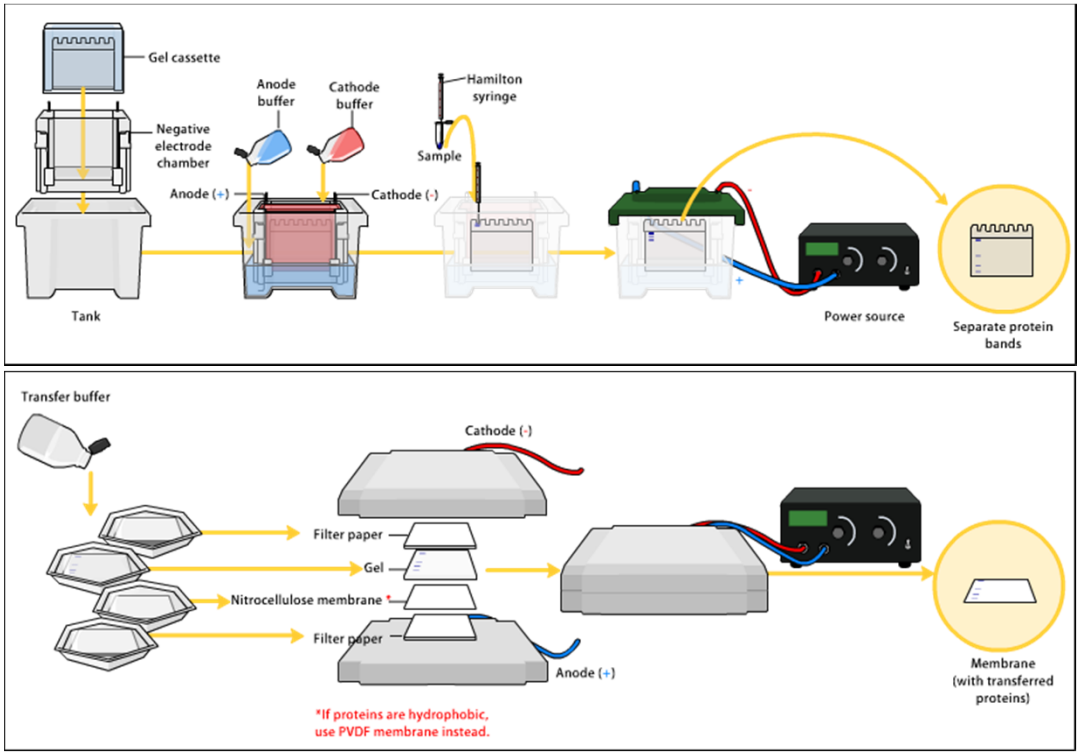
- Given a specific question, one can then come up with answers to the following three key properties of the experiment:
  1. What biological system?
     - E.g. Do I look at human cell lines, a mouse, or yeast?
  2. What perturbation/treatment conditions?
     - E.g. What compounds should I apply to the system to elicit a relevant response?
  3. What measurements?
     - E.g. What transcripts do I need to look at, and/or do I need to look at protein levels instead?
- Often (but not always), if you can't design an experiment that only has a handful of conditions and measurements, results may be difficult to interpret
  - Usually the question is too complex or not significant
  - Exceptions are screening based studies, but those also typically have a specific question of interest

Outline

- We attempt to cover many important technologies but cannot possibly cover them all.
- This Lecture—Proteins and Protein States
  - Population Average
    - Western Blotting
    - Microwestern
    - Reverse Phase Protein Array
    - Mass Spectrometry
  - Single Cell
    - Flow Cytometry
    - Mass Cytometry
    - Fluorescence Microscopy
    - Single-cell Western Blotting

Population Average

Western Blot

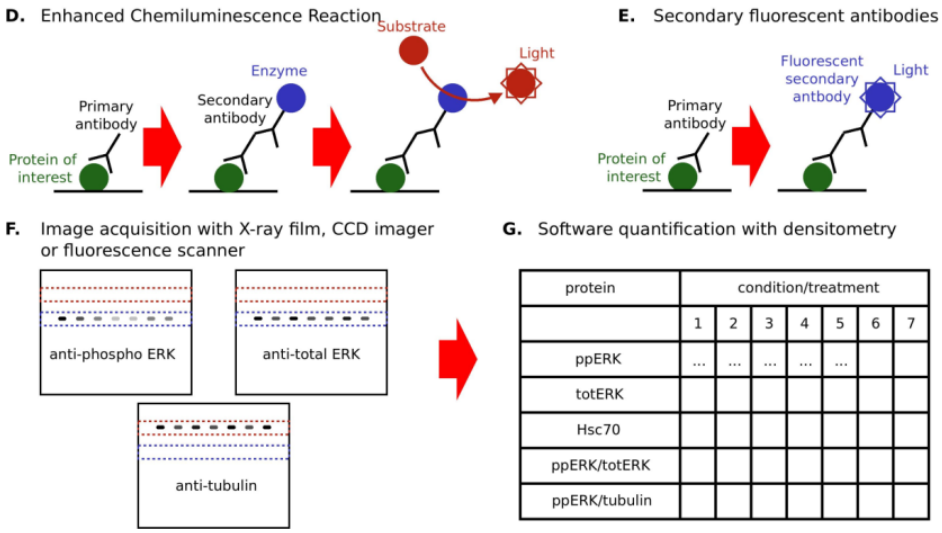


http://en.wikipedia.org/wiki/Western_blot

- Uses antibodies to visualize the levels of an epitope in a lysate

1. SDS-PAGE
   - SDS is a strong negatively charged detergent that unfolds proteins and binds to them
   - PAGE: polyacrylamide gel electrophoresis
   - This step separates proteins by molecular weight
2. Transfer
   - Take proteins separated by molecular weight out of the gel and put them onto a membrane

Western Blot—Incubation and Quantification

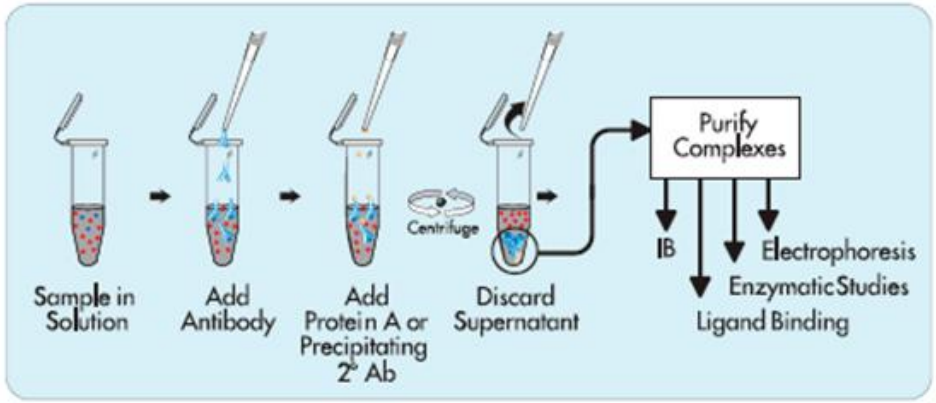


protein	condition/treatment						
	1	2	3	4	5	6	7
ppERK	...	...	...	...	...		
totERK							
Hsc70							
ppERK/totERK							
ppERK/tubulin							

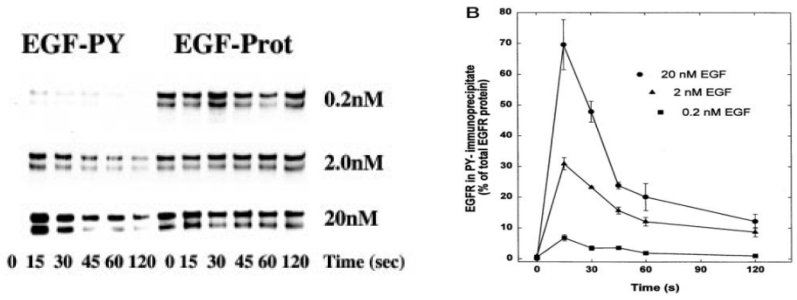
Degasperi et al., PLoS One, 2014

- Membranes are incubated with primary and secondary antibodies that allow visualization of bands
- Enhanced chemiluminescence with X-ray film is often used to detect bands, but this is only semi-quantitative
  - Density of x-ray film exposure is non-linear and quickly saturable
  - Light output of chemiluminescence reaction also has non-linear kinetics
    - Thus even detection of chemiluminesce light with a camera (e.g. CCD) can have non-linear responses
- An accepted standard for quantitative western blotting is LICOR infrared fluorescence antibodies
  - Very little background on membranes
  - Fluorescence signal is linearly proportional to amount of antibody
- Data normalization can be non-trivial (Degasperi et al., PLoS One, 2014)
  - Need to divide signal by a “loading control” that corrects for the amount of protein loaded in the gel
  - Need to normalize arbitrary signal from one membrane so it can be compared to that from another membrane.

Immunoprecipitation(IP)-Western



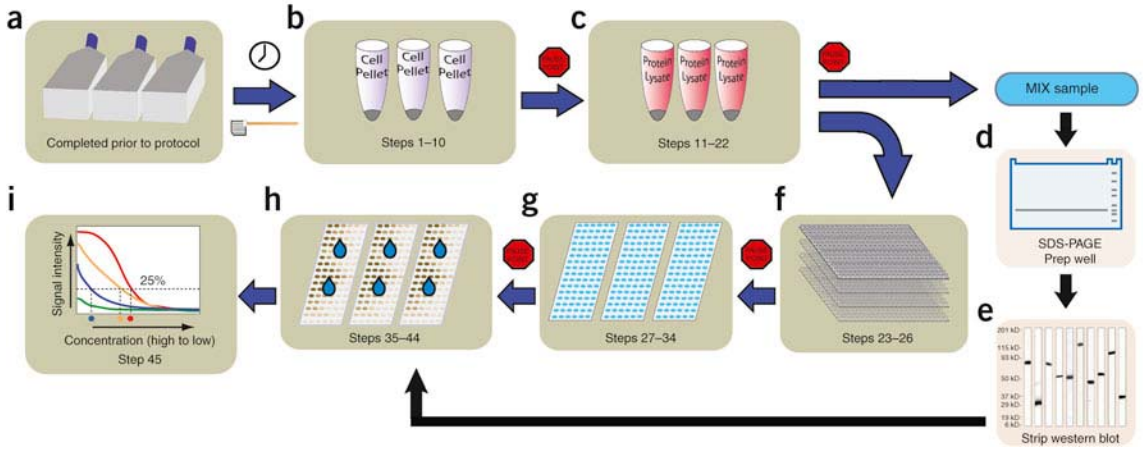
http://www.assay-protocol.com/Immunology/immunoprecipitation



Kholodenko et al., J. Biol. Chem., 1999

- Immunoprecipitation is a widely used technique where antibodies are used to "pull out" particular targets from a mixture (usually a lysate)
- Can be combined with Western Blotting in a variety of ways
- Example: quantitative % phosphorylation
  - IP the phosphorylated form of a protein of interest from a fraction of the lysate
  - Run the phospho IP alongside the total lysate or IP of the total protein in a gel
  - Blot with an antibody that recognizes both phosphorylated and total protein
    - This makes the signal comparable between the two
  - So long as volumes used along the way are recorded one can calculate the % of phosphorylated protein

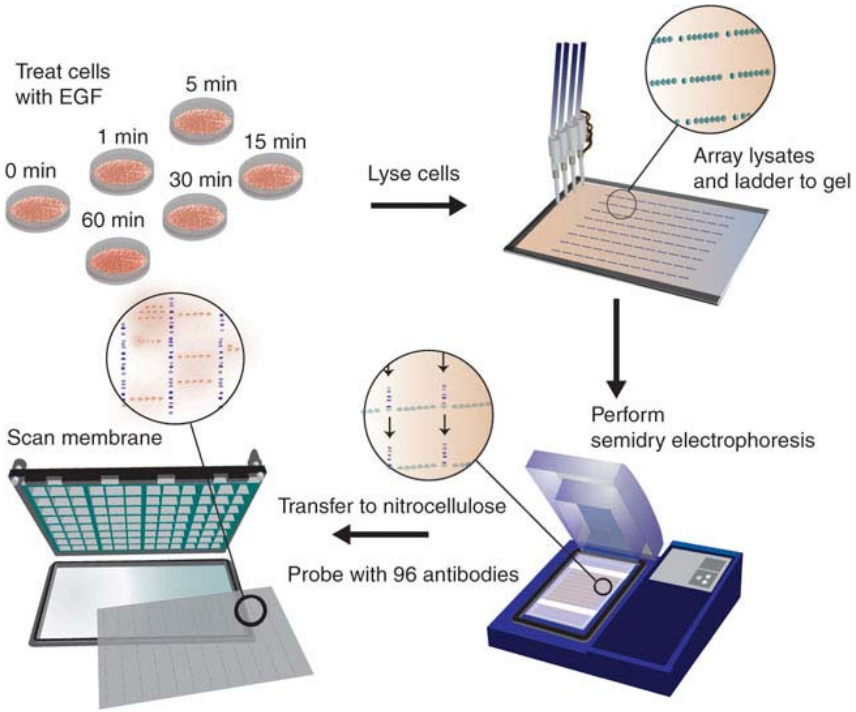
Reverse Phase Protein Array (RPPA)



Spurrier, B et al *Nature Protocols* **3**, 1796 - 1808 (2008)

- Western blot is low throughput
  - Most gels have 8-16 lanes
- One way to increase throughput is to spot many lysates onto slides (or similar) and then incubate the slides with antibodies as in western blotting
- This is the RPPA
- One can also spot many antibodies in such a way to make an antibody array
  - Available commerically
- One drawback of this approach is that antibodies must typically be of better quality than for western blotting, because proteins are not separated by molecular weight

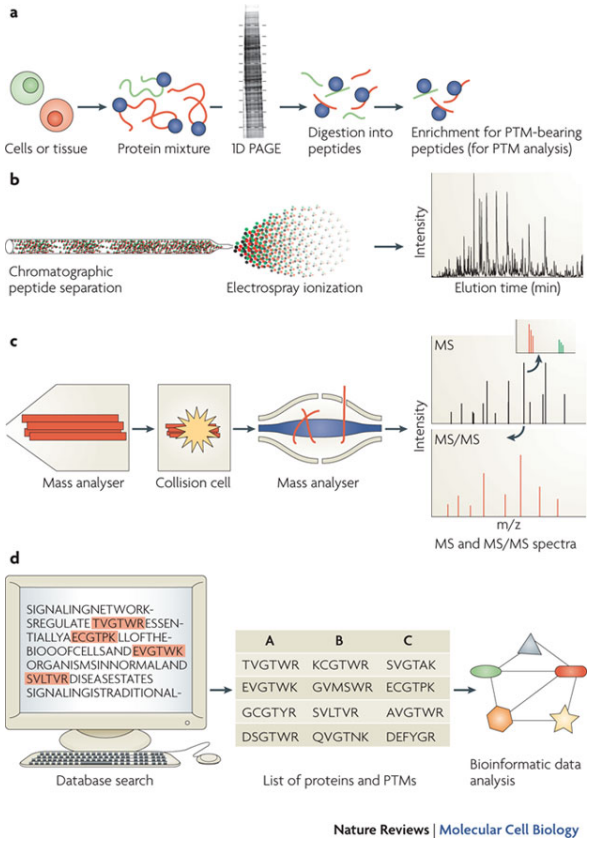
Microwestern Array (MWA)



- Western blotting is low-throughput but RPPA does not separate proteins by molecular weight
- One way to increase throughput but retain molecular weight separation is to miniaturize and automate
- The microwestern array was introduced to achieve such a goal
  - Allows parallel analysis of up to 1152 samples
    - Flexibility in number of antibodies
  - http://phosphate.uchicago.edu/Micro-Western_Arrays.html has links to excellent youtube videos on the technique from the Jones Lab at Univ. of Chicago

Ciaccio , MF et al, *Nature Methods* **7**, 148 - 155 (2010)

Mass Spectrometry

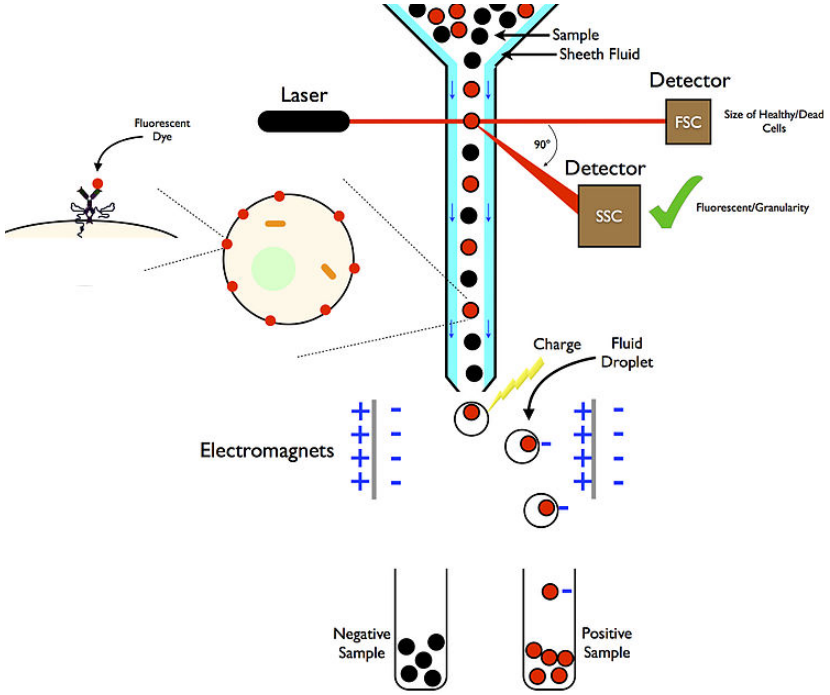


Choudhary and Mann, Nat. Rev. Mol Cell Biol, 2010

- We will cover mass spectrometry in much greater detail in a later week.
- Called "proteomics" when it is applied to proteins, since it can be used in a somewhat unbiased way to look at many proteins within a sample
  - Also used to look at metabolites→metabolomics
  - Proteomics almost always involves tandem mass spectrometry (two mass specs in parallel)
- Can be used to measure, for example,
  - Total levels of proteins
  - Levels of post-translational modifications
    - Phosphorylation, hydroxylation, glycosylation, etc….anything with a mass can essentially be measured
  - Protein-protein interactions when combined with IP
- Typically involves
  - Separation
    - Simplify the mixture of proteins
  - Digestion
    - Turn the proteins into smaller peptides
  - Enrichment and More Separation
    - Select for certain post-translational modifications (if desired), chromatography
  - Ionization
    - Give the peptides a charge so that they can be analyzed by mass spectrometry
  - Mass Filtering (MS1)
    - Select peptides with a particular mass to charge ratio
  - Fragmentation and mass analysis (MS2)
    - Break the selected peptides apart and measure the masses of the pieces
    - This fragmentation gives a "fingerprint"
  - Identification
    - Determine which protein the fragments came from by amino acid sequence analysis
  - Quantification
    - Relate the number of detected peptides to the amount of parent protein in the original sample
- There are a variety of ways in which these steps can be done and we will cover some of the major ones in detail later.

Single Cells

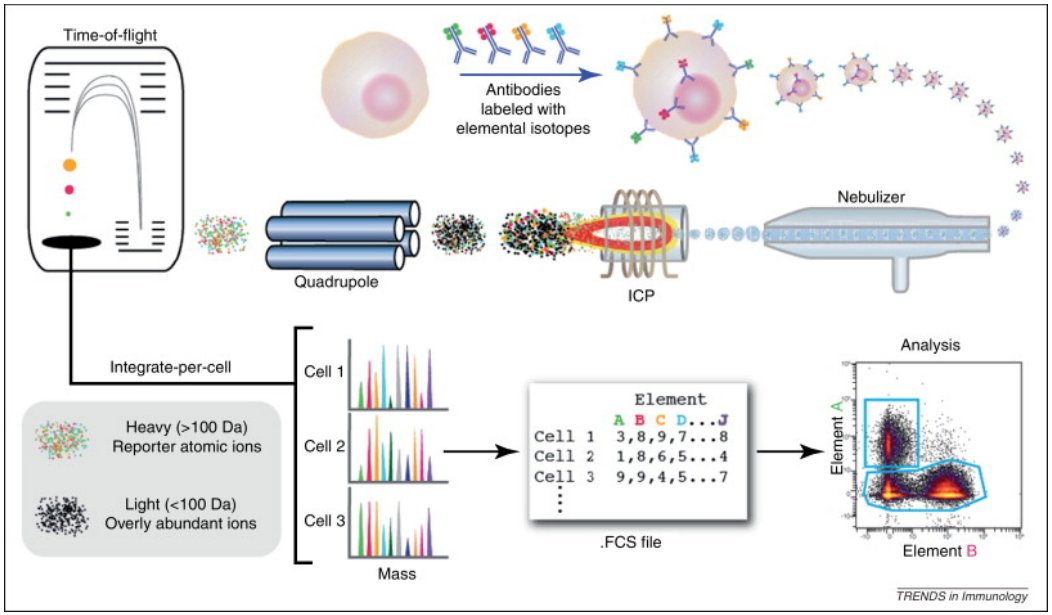
Flow Cytometry and Flourescence Activated Cell Sorting (FACS)



http://en.wikipedia.org/wiki/Flow_cytometry#mediaviewer/File:Fluorescence_Assisted_Cell_Sorting_%28FACS%29_B.jpg

- We will study this in detail in a later week
- Cells are labeled with fluorescent probes (e.g. antibodies with fluorophore tags)
  - Live or fixed cells
- Labeled cells in suspension are flowed past a laser(s) and fluorescence is quantified in single cells
- It is possible to sort cells based on the observed fluorescence (FACS)

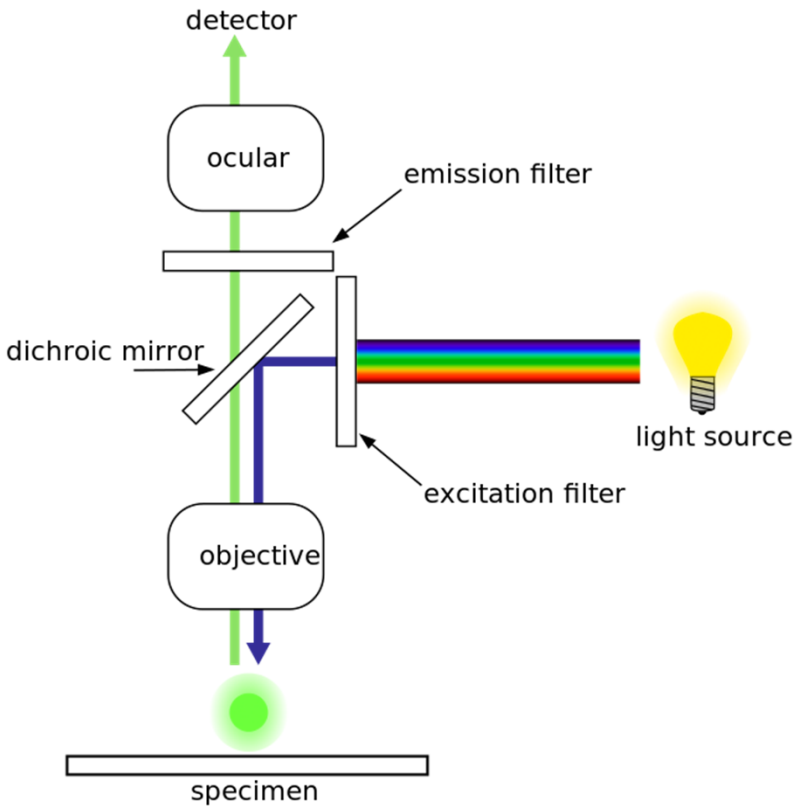
Mass Cytometry



Bendall, SC et al., *Trends in Immunology*, **33** 323-332 (2012)

- Recent advance which uses mass tagged antibodies instead of fluorescent tagged antibodies
- A flow cytometer coupled to a time-of-flight mass spectrometer (CyTOF)
- Enables a much higher degree of multiplexing due to availability of independent mass tags
  - ~40s
  - Mass tagged antibodies can also be used in immunostaining (Giesen et al., Nat Methods, 2014)
- We will also study this in more detail in coming weeks

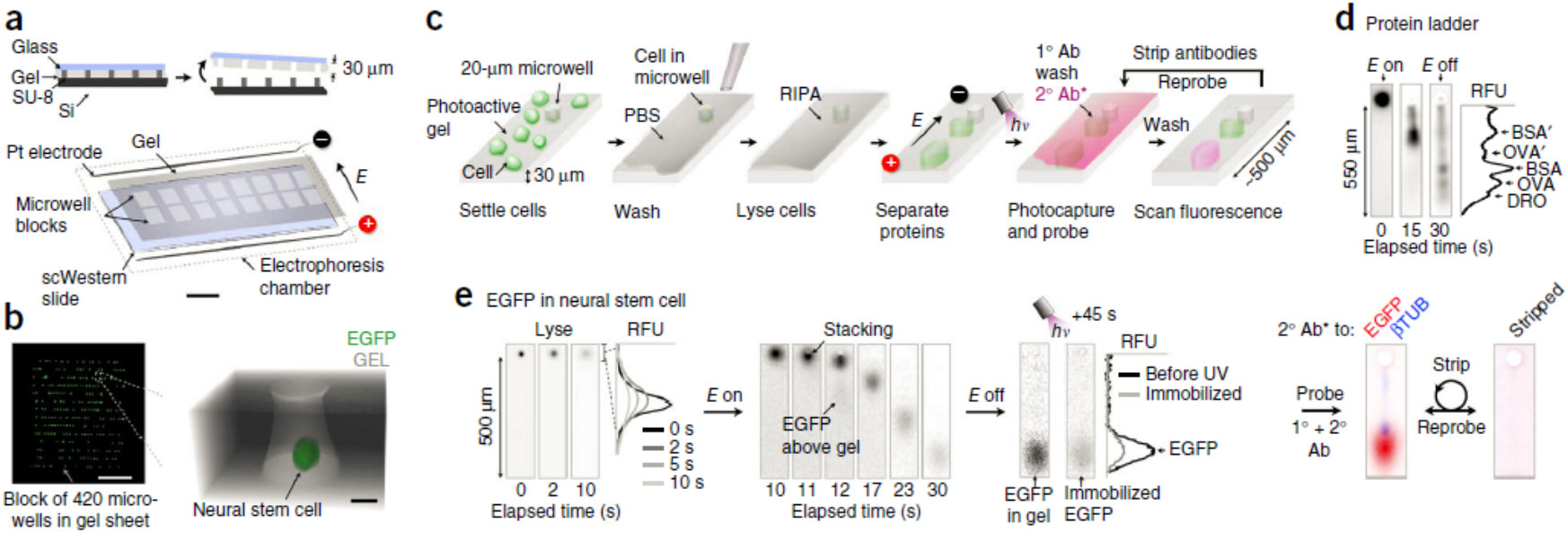
Fluorescence Microscopy



http://en.wikipedia.org/wiki/Fluorescence_microscope#mediaviewer/File:FluorescenceFilters_2008-09-28.svg

- Immunofluorescence
  - Fixed cells
  - Similar to western or flow/mass cytometry in that it relies on antibodies
- Live-cell imaging
  - Repeatedly image the same cells over a time course
  - Variety of sensors
    - Small molecules
      - E.g. mitotracker that stains mitochondria
    - Genetically encoded
      - Plasmids transfected into cells or they stably express
      - Localization of fluorescence
      - Förster Resonance Energy Transfer between two fluorescent proteins
        - » Inter or intramolecular
- Can be applied in a traditional microscope or in a high throughput/high content setting
- Will be studied in detail in Week 6

Single Cell Western Blot



Hughes et al., Nat Methods, 7(11), 2014