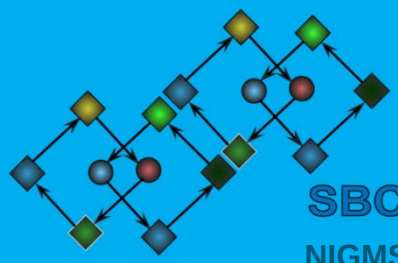


NIGMS funded Center

Experimental Methods in Systems Biology

Part of the Coursera Certificate in Systems Biology

Marc Birtwistle, PhD
Department of Pharmacology & Systems Therapeutics
Fall 2014, Week 1, Experimental Perturbations

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

- "Basal" Perturbations
  - Genetic
  - Non-genetic
- "Acute" Perturbations
  - Compound-based (pharmacological and biological)
  - Light-based
- We focus on mammalian cell line and cell culture studies, but many of the techniques we describe can be applied to other model systems (with reasonable alteration)
  - There are lots of techniques in mouse genetics that I won't cover much here

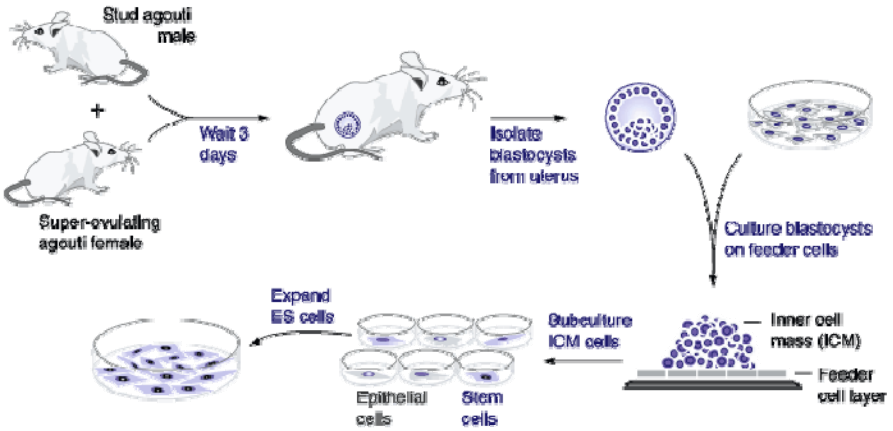
Basal vs. Acute Perturbations

- Basal Perturbation: An alteration that is permanently present (or at least can be considered permanent relative to the time scale of the experiment), and for which one does not measure dynamic response.
  - Often followed by an acute perturbation
  - Can also be thought of as “chronic”

- Acute Perturbation: An alteration to the biological system that induces rapid changes (relative to the time scale of the experiment)
  - One is often interested in a specific time point or an entire time course of many time points post-perturbation

Basal Perturbations

Genetic—Mouse Embryonic Fibroblasts (MEFs) or Embyronic Stem (ES) Cells

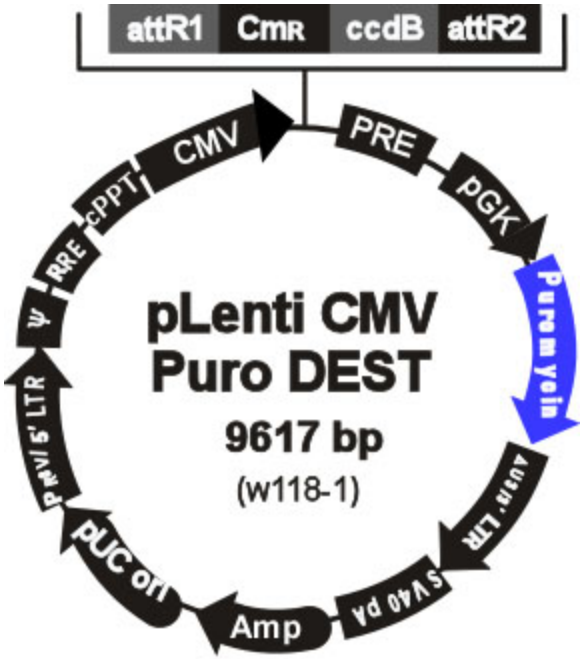


http://www.biochem.arizona.edu/miesfeld/teaching/Bioc471-2/pages/Lecture19/Lecture19.html

- Wide variety of mouse genetic knockouts are available
- Many knockouts are embryonic lethal, but one can usually at least obtain fibroblasts or ES cells from the embryo
  - They can be grown in culture
- These can be useful for exploring the molecular functions of particular gene products
- They can also be useful for negative and positive controls for other types of experiments
- ES cells can be differentiated into a variety of other cell types (growing field)
  - Induced pluripotent stem (iPS) cells are similar but can be derived for adult human cells (e.g. fibroblasts)

Xu, Current Protocols in Molecular Biology, Unit 28.1 2005

Genetic—Plasmid-Based Selection

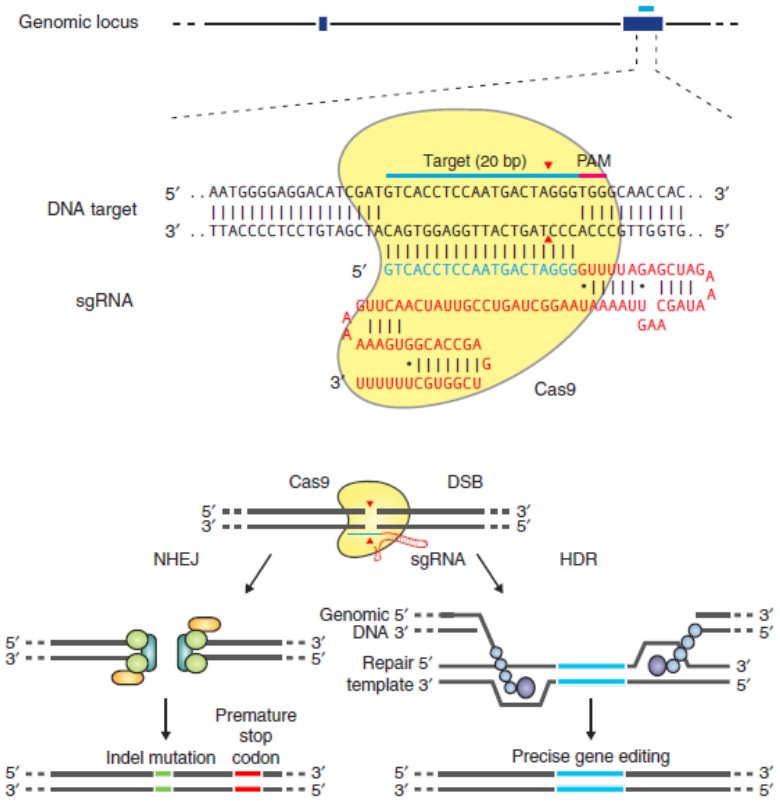


https://www.addgene.org/static/data/61/90/2513a6d2-af62-11e0-90fe-003048dd6500.jpeg

- Plasmids can contain "resistance genes"
  - Code for a protein that metabolizes a toxic drug
  - Puromycin, G418 (similar to neomycin), blasticidin, zeocin, hygromycin B
- By transfecting the plasmid and treating cells with the drug, only cells that incorporated the plasmid's resistance gene will survive
  - The plasmid gets randomly incorporated into the genome
  - Linearizing the plasmid by making a single restriction enzyme cut improves efficiency
- Incorporating the plasmid into a lentivirus can improve efficiency
- One can also use fluorescent proteins combined with flow sorting as an alternative (although there is no active selection pressure)

Campeau et al., PLoS One, 2009

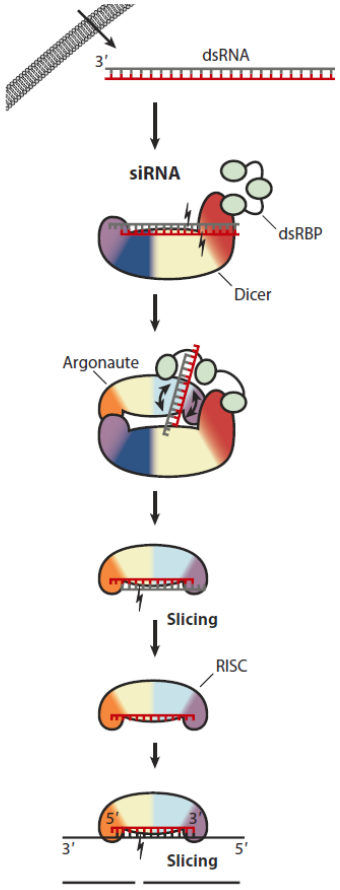
Genetic—Genome Engineering



Ran et al., Nat Protocols, 2013

- Genome engineering: directed modification of particular loci
- General strategy—induce DNA double strand breaks and then rely on
  - non-homologous end joining for repair, which typically disrupts gene function
  - Homologous recombination, which places a provided double stranded DNA template where the break occurred
- Recombination enzymes
  - Flp recombinase and CRE/Lox systems—these recombination enzymes
- TALENs and ZFNs
  - Requires protein engineering to achieve DNA sequence specificity of ~12-24 bp and do not always cut in the same location
  - Can only reasonably target one locus at a time.
  - Thus they are somewhat difficult to use and target
- Latest—CRISPR/Cas9 (Ran et al., Nat Protocols, 2013 and References therein)
  - Uses a 20 bp “guide RNA” (gRNA) to bring the Cas9 nuclease to DNA which is complementary to the guide RNA
    - Allows simple and cheap targeting by oligo synthesis
    - Can be targeted almost anywhere in the genome quite specifically (20 bp can be reasonably unique)
    - Tools at www.genomeengineering.org
  - Wild type Cas9 vs. “nickase” Cas9
    - Wild type catalyzes a full double strand break where the gRNA dictates
    - Nickase Cas9 catalyzes a single strand break, therefore one needs two “nicks” to make the double strand break

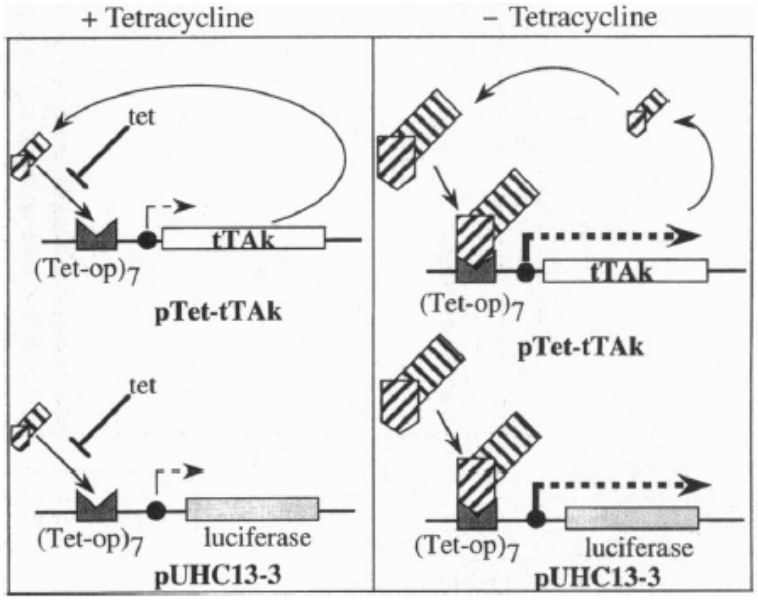
Non-Genetic—RNA interference



Wilson and Doudna, Ann. Reviews Biophysics, 2013

- Discovered in 1998 by Fire and Mello (Fire et al., Nature, 1998)—awarded Nobel prize in 2006
  - Notably rapid progression from discovery to Nobel
- A short (~20 bp) duplex RNA is directed to complementary transcripts, which are then degraded
  - Duplex can be small interfering RNA (siRNA)—two independent strands, or short hairpin (shRNA)—one strand that hybridizes to itself with a stem loop at one end (looks like a hairpin!)
- This "knocks down" expression of the corresponding genes
  - Similar pathways process micro RNAs (miRNA) that are produced endogenously and can inhibit translation and degrade mRNAs
- Efficiency of knockdown widely varies across genes and cell types
- Need to use two independent sequences to control for off-target effects
  - Gold standard is a "rescue" experiment, where an RNAi resistant transcript is ectopically expressed from a plasmid (resistant through engineered silent mutations)
- Can be done in a transient or stable manner
- Can be inducible (see next slide)
- Collection of Reviews: http://www.nature.com/focus/rnai/index.html

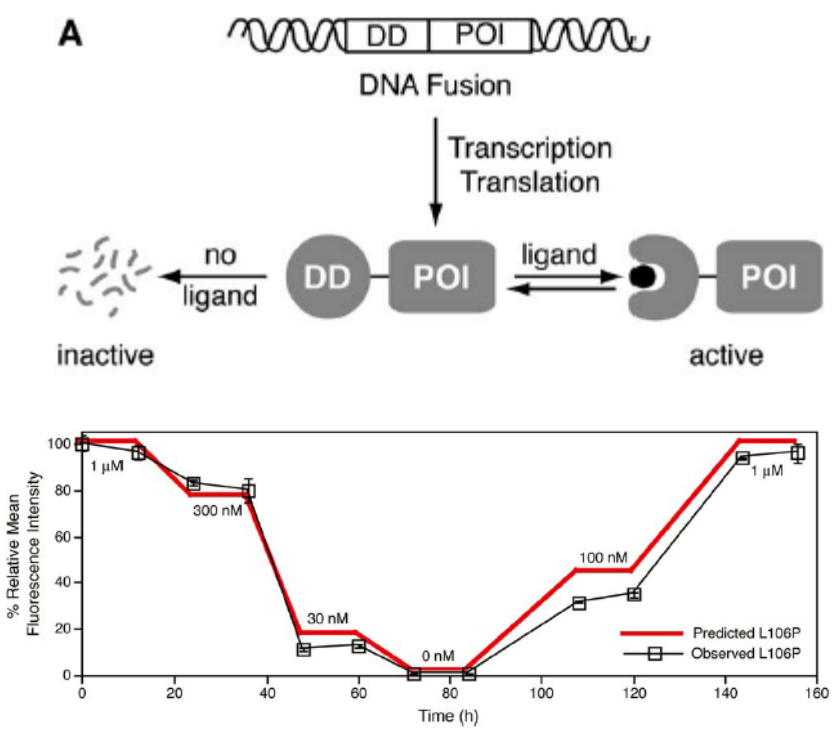
Non-Genetic—Transcription Rate



- A variety of systems exist to control transcription rate with small molecules
  - Too many to cover here, but they all work similarly to a certain extent
- One common system is with tetracycline (or more commonly its more stable and higher affinity analog doxycycline)
- Tet-on system:
  - Cells expressing the tet repressor, and with the gene of interest downstream of the tet repressor binding sites
  - The tet-repressor only binds DNA when it is not bound to tetracycline
- There are also “Tet-off” systems
- Can control expression of genes and shRNAs

Shockett et al., PNAS, 1995

Non-Genetic—Protein Half-Life



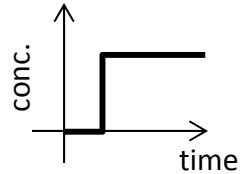
- A few systems allow modulation of protein half life with small molecules
- They all involve some kind of alteration to the protein-of-interest
- Examples
  - DD domain/Shield1 (Banaszynski et al., Cell, 2006; 126(5): 995-1004)
    - Shield1 enters live cells, binds to the DD domain, and increases protein stability.
  - auxin/AID (Holland et al., PNAS, 2012; 109(49))
    - Auxin (IAA) works similar to Shield1 but decreases protein stability
    - However it requires ectopic expression of TIR1

Banaszynski et al., Cell, 2006; 126(5): 995-1004

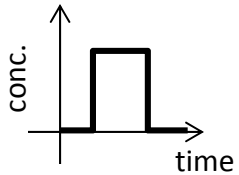
Acute—Pharmacological or Biological Compounds—Pipette-Based

- Pharmacological: a compound that is not naturally occurring in the system of interest but interacts with the model system of interest
  - E.g. a drug
- Biological: a compound that normally interacts with the system of interest in a biological context
  - E.g. a growth factor

Step Input: Treatment with a compound at a certain constant concentration.



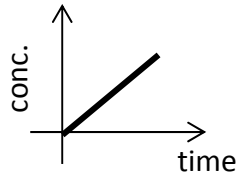
Pulse-Chase Input: Treatment with a compound at a certain concentration for a specified time period, followed by a second concentration (usually zero).



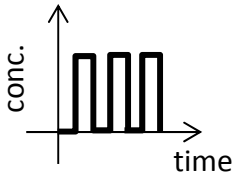
Acute—Pharmacological or Biological Compounds—Pump-Based

- Although it is not common or very simple, one can use perfusion or pump-based systems to create complex time dependent inputs
- Microfluidic designs can give enormous flexibility here

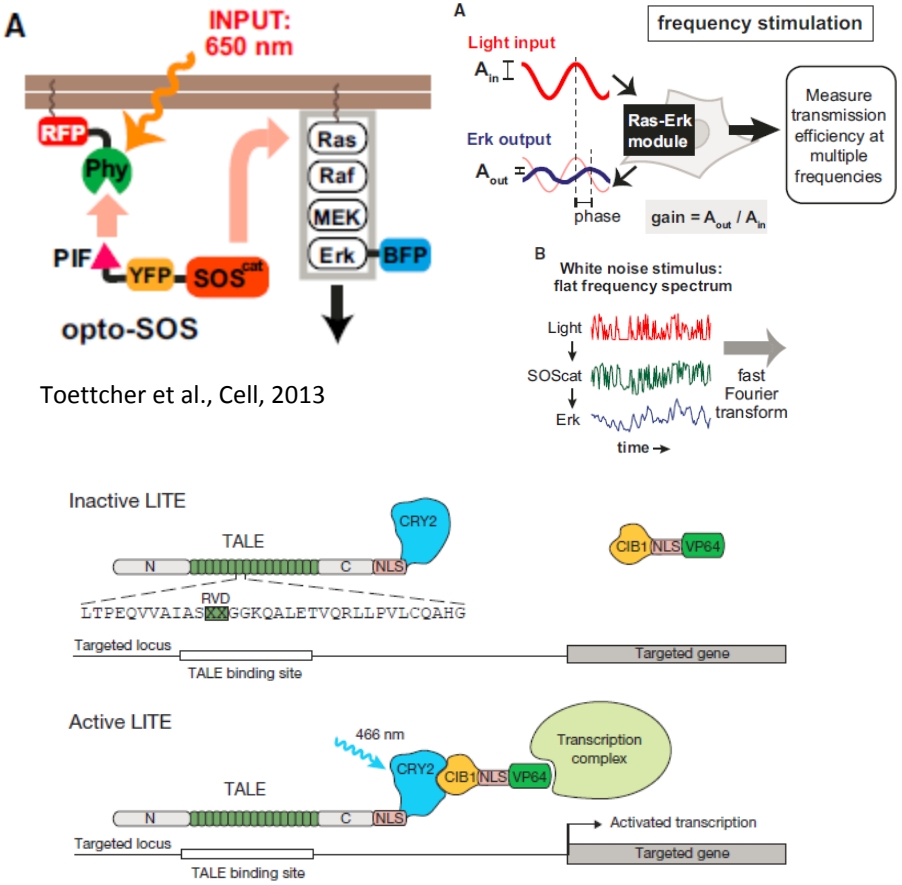
Ramp: Treatment concentration linearly ramps up or down with time (e.g. Sasagawa et al., Nat. Cell Biol., 7, 2005)



Wave: Treatment concentration oscillates in time with a fixed amplitude and period (e.g. Mettetal et al., Science, 2008, 319 (5862)



Acute—Light-Based



Toettcher et al., Cell, 2013

Konermann et al., Nature, 2013

- Optogenetics: Using light-sensitive proteins to control biochemical activities.
- First application was ion channels in neuroscience (reviewed in Zhang et al., Cell, 2011, 147(7))
- Has been extended to various light-sensitive protein-protein interactions (e.g. Levskaya et al., Nature, 2009—Phy-PIF; Kennedy et al., Nat. Methods, 2010—CIB-CRY) and transcription (Konermann et al., Nature, 2013)

Next Time—Nucleic Acid Measurements