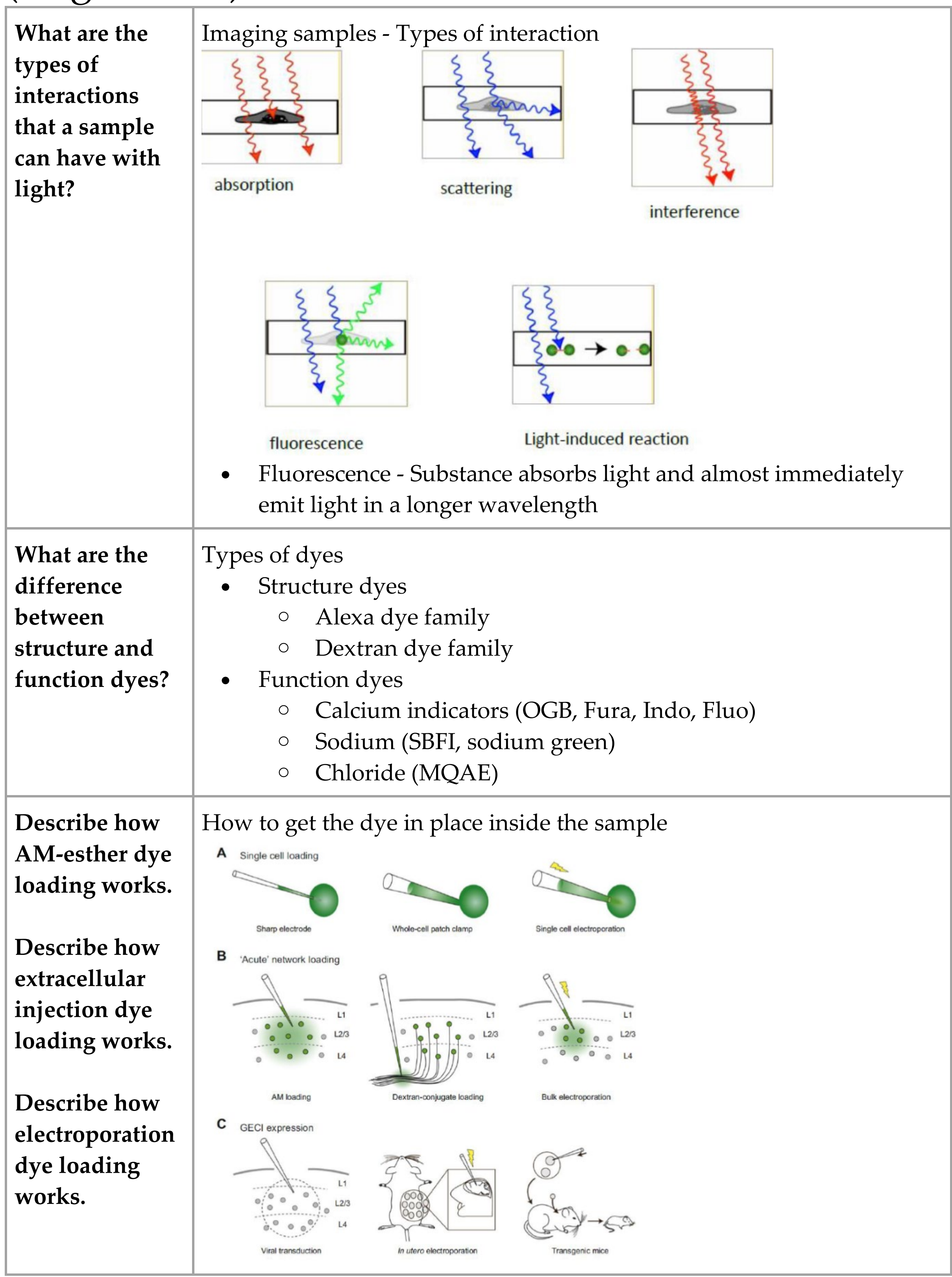
Introduction to Fluorescence Microscopy - Dyes (Rogier Min)



What are the types of interactions that a sample can have with light?	Imaging samples - Types of interaction • Fluorescence - Substance absorbs light and almost immediately emit light in a longer wavelength
What are the difference between structure and function dyes?	Types of dyes • Structure dyes ○ Alexa dye family ○ Dextran dye family • Function dyes ○ Calcium indicators (OGB, Fura, Indo, Fluo) ○ Sodium (SBFI, sodium green) ○ Chloride (MQAE)
Describe how AM-esther dye loading works. **Describe how extracellular injection dye loading works.** **Describe how electroporation dye loading works.**	How to get the dye in place inside the sample

<table>
<tr>
<td></td>
<td>

- AM-Esther dye loader - Lypophilic shell around dye; once it enters the cell, the AM esther is cleaved by estherases present in the cell, dye only then starts to fluoresce and it cannot leave the cell
- Extracellular injection - Endocytosis/Pynocytosis cause the dye to get inside the cell
- Add dye to pipette solution during patch clamp
- Electroporation - Dyes are often charged, when you shock the membrane it becomes more porous and the dye is able to get in
  - Can be done with a single cell or many cells at a time

</td>
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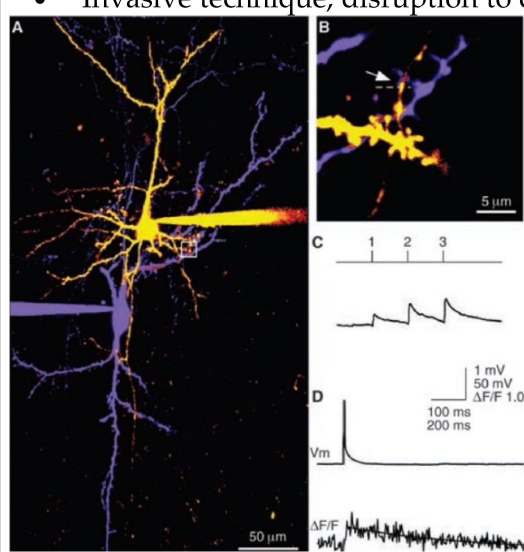
<tr>
<td>What are the advantages and disadvantages of inserting the dye via intracellular recording?</td>
<td>

1)Intracellular recording

- Single cell resolution, fill all cell compartments
- Invasive technique, disruption to cell membrane



Dendrites ramification into 2 equidistant branches, while axons tend to branch into more abrupt angles

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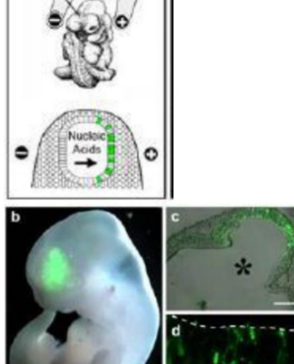
<tr>
<td>What are the advantages and disadvantages of inserting the dye via electroporation?</td>
<td>

2. Electroporation



- Single or multiple cell, can be done with different cell populations
- Invasive technique with precise timing required

</td>
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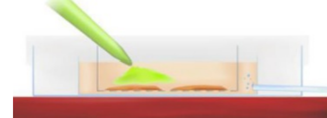
<tr>
<td>What are the advantages and</td>
<td>

3. Bulk loading

</td>
</tr>
</table>

disadvantages of inserting the dye via bulk loading?

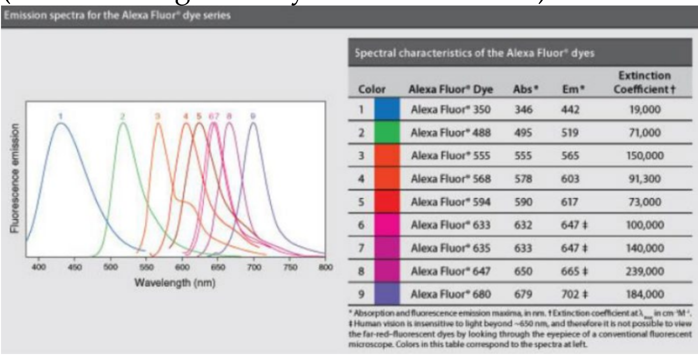


- Multiple cell loading, easy application
- Background staining can obscure fine processes, variable intracellular concentrations, DMSO and detergents are used in loading; Tends to work better with tissues from young animals compared to older animals

What are the main dyes used for structural imaging?

Dyes for structural imaging

- Fluorescein
- Alexa dye - Sulphonated forms of dyes including rhodamine and fluorescein; Quite stable and bright, high extinction coefficient (how much light the dye is able to absorb)

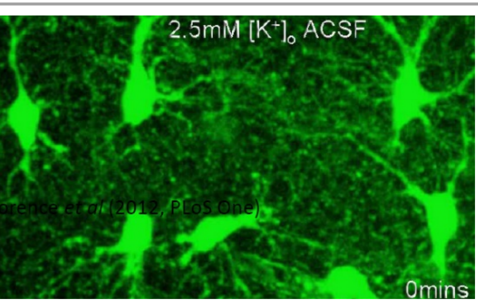


Spectral characteristics of the Alexa Fluor® dyes

Color	Alexa Fluor® Dye	Abs*	Em*	Extinction Coefficient†
1	Alexa Fluor® 350	346	442	19,000
2	Alexa Fluor® 488	495	519	71,000
3	Alexa Fluor® 555	555	565	150,000
4	Alexa Fluor® 568	578	603	91,300
5	Alexa Fluor® 594	590	617	73,000
6	Alexa Fluor® 633	632	647 ‡	100,000
7	Alexa Fluor® 635	633	647 ‡	140,000
8	Alexa Fluor® 647	650	665 ‡	239,000
9	Alexa Fluor® 680	679	702 ‡	184,000

* Absorption and fluorescence emission maxima, in nm. † Extinction coefficient at λ_{max} in $cm^{-1}M^{-1}$. ‡ Human vision is insensitive to light beyond ~650 nm, and therefore it is not possible to view the far-red–fluorescent dyes by looking through the eyepiece of a conventional fluorescent microscope. Colors in this table correspond to the spectra at left.

- Dextran dyes
- Complex polysaccharide used for cell structure visualization and also blood vessel/membrane permeability
- Allows for the visualization of climbing fibers (dye moves along axons)
- Can be injected into vasculature - Since it is a big molecule, it never leaves the blood vessels
- Sulforhodamine-101



- Dye that is especifically taken up by astrocyte (not neurons) - Possibly due specific pumps only expressed in astrocytes

What is quantum yield?

Quantum yield - For each photon it absorbs, how many photons it emits back (values closer to 1 indicate brighter dyes)

Dye[a]	MIF_{corr}[b]	$logD_{hyd}$[c]	$logD_{unhyd}$[c]	λ_{max} (ex)	λ_{max} (em)	ε (M^{-1} cm^{-1})[d]	QY[d]
Abberior STAR 635P azide	0.21±0.02	n/a	0.58	634	654	80000	0.55
Alexa 488 SE	−0.003±0.007	−11.09	−8.02	494	517	73000	0.92
Alexa 532 SE	0.04±0.01	−3.26	−0.16	530	555	81000	0.61
Alexa 532 M*	0.58±0.05	−3.61	0.13	528	552	78000	0.61
Alexa 546 SE	0.18±0.03	−3.68	−1.43	554	570	112000	0.79
Alexa 555 M	0.04±0.03	–	–	556	572	158000	0.1
Alexa 568 hydrazide	0.04±0.01	n/a	−5.89	576	599	86000	0.69
Alexa 594 M	0.3±0.1	−7.4	−3.66	588	612	96000	0.66
Alexa 633 M	8.0±0.5	−3.44	0.3	622	640	143000	–
Alexa 647 SE	0.03±0.02	−6.72	−3.72	651	672	270000	0.33
Alexa 647 M	0.04±0.02	−8.1	−4.26	651	671	265000	0.33

Why is calcium a good ion to analyse cellular function?

Relevant ion concentration gradients

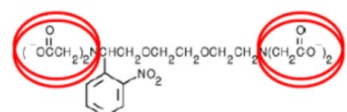
- Cells have high potassium, low sodium, low chloride and very low calcium
    - Calcium is used for signaling because of this very low concentration - Small changes in concentration can be easily sensed (why calcium dyes are so popular)
- Kd - Dissociation constant (lower number -> higher affinity and saturates faster

How are most calcium indicators made?

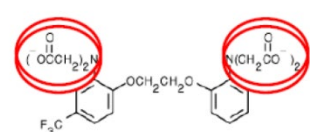
Calcium indicators

- Used based on EGTA/BAPTA are present in the molecules as ionophores (groups that bind to calcium)

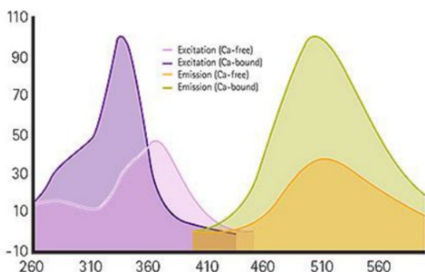
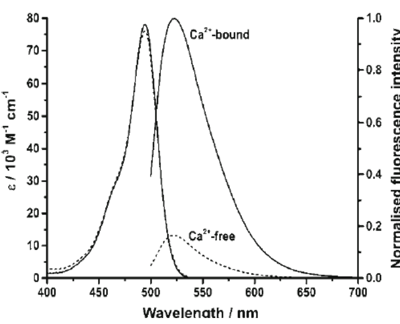
EGTA



BAPTA

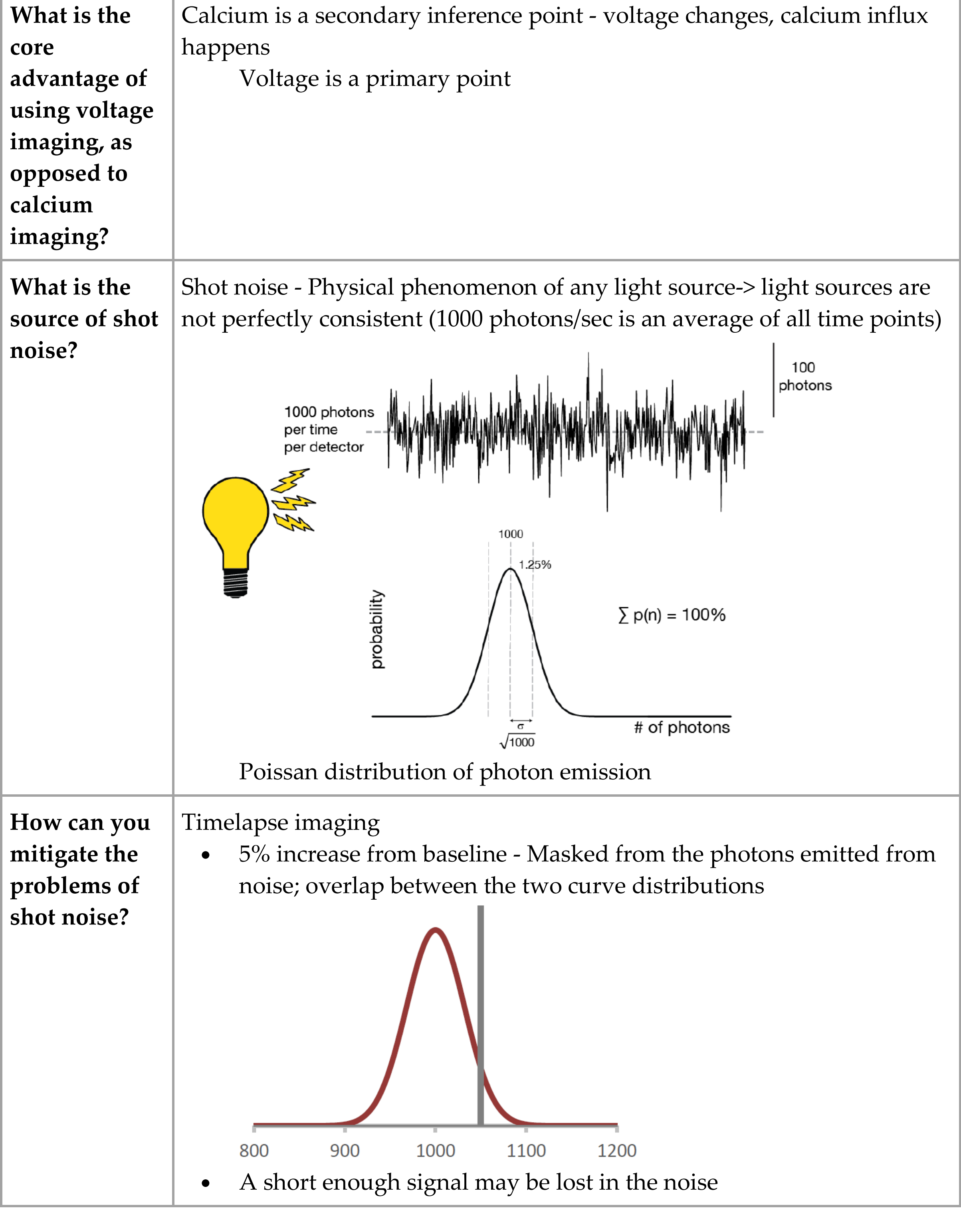


- These ionophores are bound to fluorophores in order to make the calcium indicators

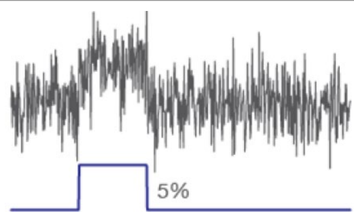
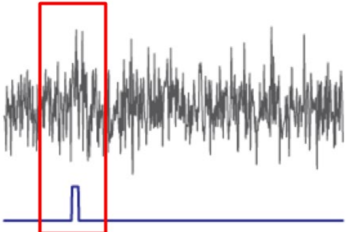
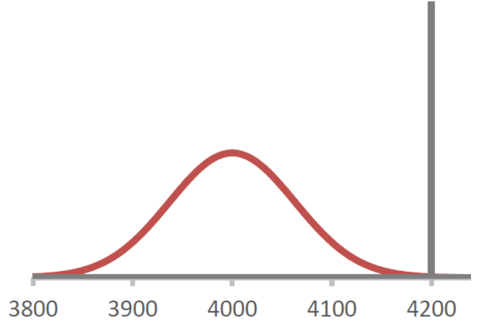
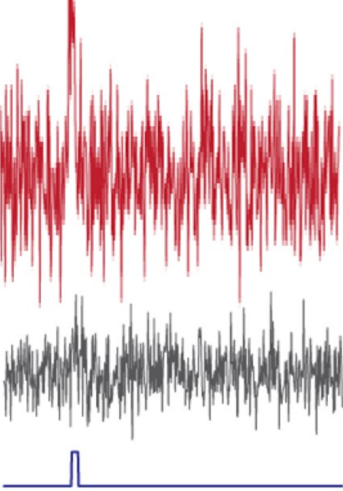
What are radiometric indicators?	Radiometric indicators - Calcium binds leads to shift of electrons from the fluorophore to the ionophore -> Shift energy levels of ground/excited states  ○ Ratio of these two wave lengths can be used to analyse activate/inactive molecules -> can be directly related to calcium concentration if the system is calibrated well
What are intensiometric indicators and how do they compare with ratiometric indicators?	Intensiometric indicators - Calcium free ionophore quenches the fluorescence of the fluorophore when there is no calcium, when calcium binds, the quenching decreases and the fluorescence intensity increases  Ratiometric indicators allow for better and easier calibration Ratiometric indicators require multiple excitation/emission pathways, intensiometric indicators require just a single set
What are the main advantages of modern fluorescence dyes compared to 100 years ago?	Advantages of modern fluorescence dyes compared to 100 years ago • Visualizing living cells - No fixation artefacts • Visualizing dynamic morphological changes - Resolution of single molecules, and miliseconds • Visualizing cells in vivo - The same neuron/dentrite can be followed for months • Trace long-range projecting axonal pathways • Distinguish multiple cellular types
What are the advantages of fluorescent dyes compared to other techniques?	Disadvantages of fluorescent dyes • Can influence the decay time of calcium signals (buffer effect) ○ Neuron naturally have buffering proteins (PV), but an additional buffer may lead to a disturbance of normal processes • Can increase spatial spread of calcium - The dyes are much more mobile than endogenous buffering proteins

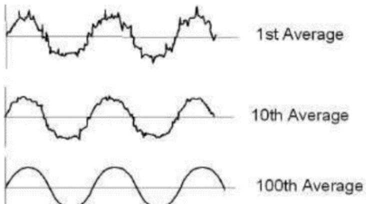
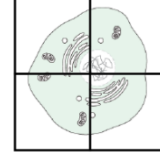
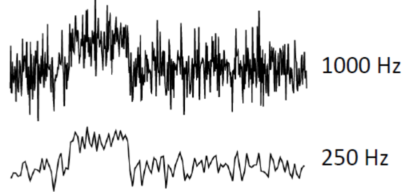
	• Can be toxic (byproducts of de-esterification) - May damage the cells • Can compartimentalize inside the cell - Clumps may be bound to organelles • Unwanted effects - E.g. Fluorescent Ca^{2+} indicators inhibit Na-K-ATPase

Introduction to voltage imaging (Marko Popovic)



What is the core advantage of using voltage imaging, as opposed to calcium imaging?	Calcium is a secondary inference point - voltage changes, calcium influx happens Voltage is a primary point
What is the source of shot noise?	Shot noise - Physical phenomenon of any light source-> light sources are not perfectly consistent (1000 photons/sec is an average of all time points) Poissan distribution of photon emission
How can you mitigate the problems of shot noise?	Timelapse imaging • 5% increase from baseline - Masked from the photons emitted from noise; overlap between the two curve distributions • A short enough signal may be lost in the noise

	  ○ How to overcome this? Increase light intensity /number of photons • Your shot noise distribution is flattened out -> smaller signals are more away from the center of noise distribution  • Photon count increases linearly and noise increases with the square root of the photon count 
What are the possible ways	Increasing light intensity

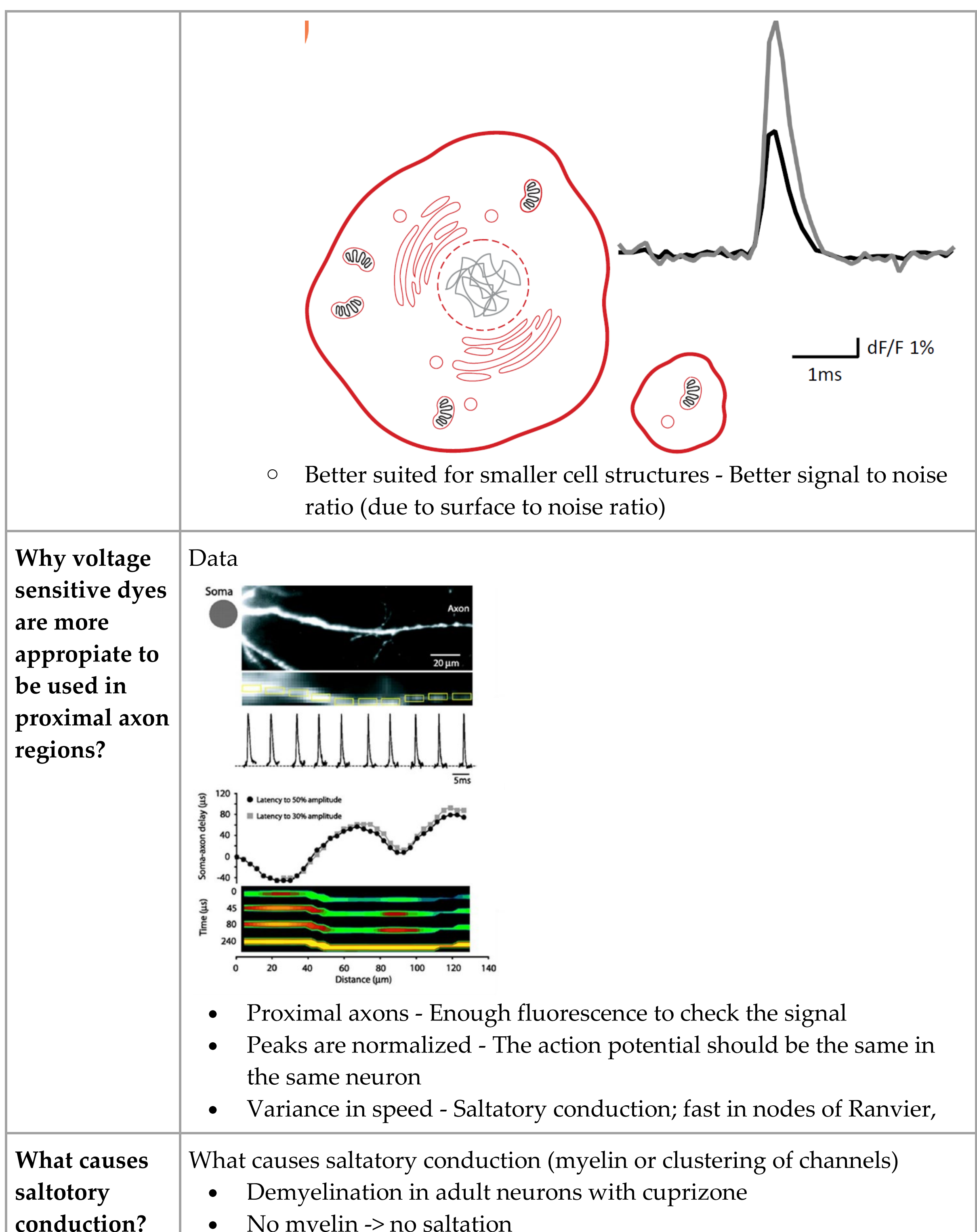
to increase light intensity?	• Phototoxicity - Reactive oxygen species damage the cell; solution - short bursts of imaging
	• Trial averaging or accumulation - not possible with random events
	  ○ Signals need to be aligned/very well describe - Such as an action potential
	
	
	• Reduce spatial resolution - Reduce magnification
	  ○ Each individual pixel is exposed to more light
	
	• Reduce temporal resolution - Reduce detection rate
	  ○ Still needs to be good enough to identify fast processes
	
	
	• Optimize light path
	  ○ High quality optics with the highest possible NA
	  ○ The higher NA, the shorter the imaging distance

What are other sources of noise, aside from shot noise?	Other sources of noise
	• Excitation light noise
	  ○ Fluctuations of your lamp, the quieter the light source the better
	• Vibration noise
	  ○ If your specimen is moving around the same
	  ○ Needs an antivibration table
	• Read noise
	  ○ Photons per time per detector - CMOS detector have really good read noise

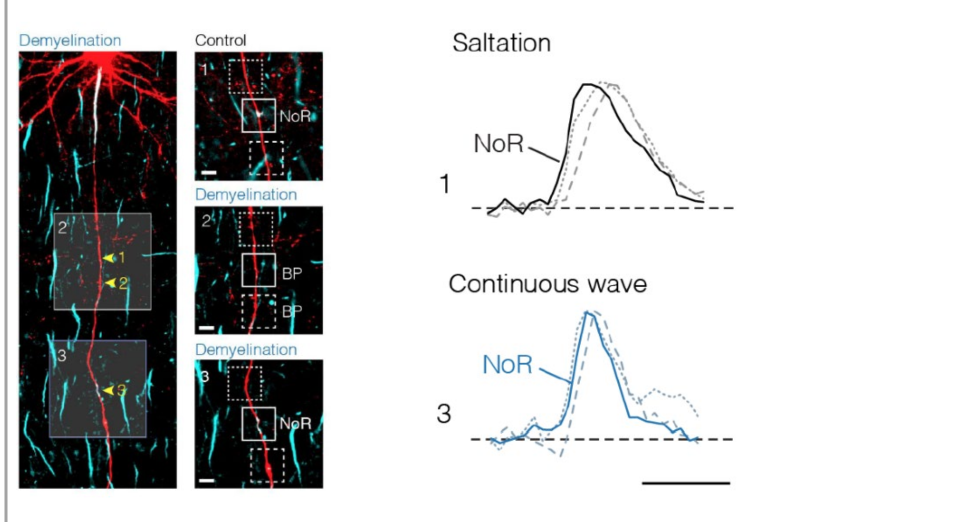
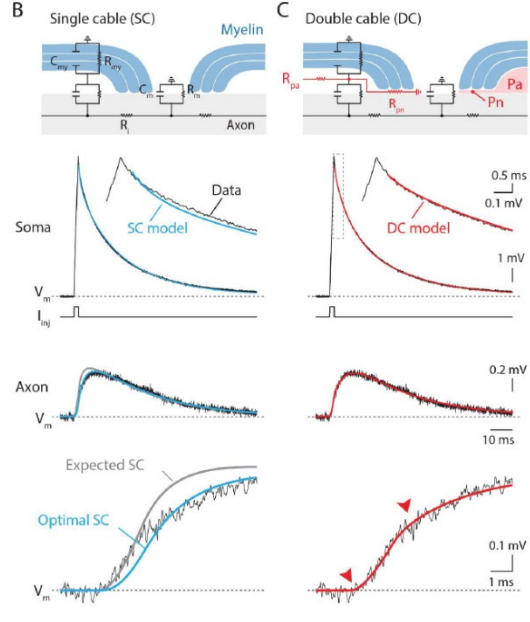
What are the main things to do in order to get the best possible image?	Imaging tips (Summary) Maximize the light intensity to maximise signal to noise ratio Signal rises linearly with light and noise rises with sqare root of light To get more light intensity you have to compromise something (resolution, speed or add phtotoxicity/bleaching) Choose an appropriate resolution to capture your object at a Nyquist rate without sacrificing light intensity!

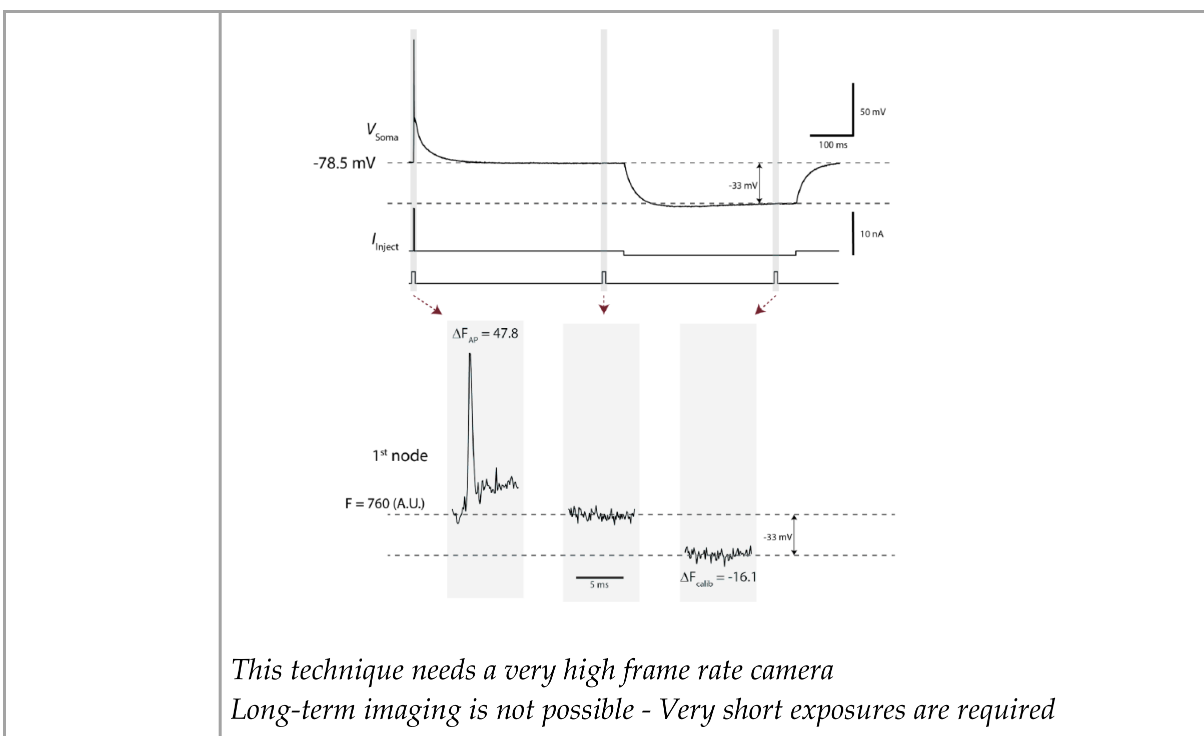
<table>
<tr><td>Why use voltage imaging, instead of using electrodes?</td><td>Measuring membrane potential transients (why use voltage instead of electrodes?)
• You cannot use 30 electrodes in one cell
• Some processes are really thin and difficult to patch</td></tr>
<tr><td>What was the main shortcome of the first voltage sensitive dye?</td><td>Voltage sensitive dye imaging (VSDI)
• Line - Electric signal
• Dots - Optic signal (varied because of shot noise)
• This used absorbance - Nowadays, we use fluorescence in science (better sensitivity)</td></tr>
<tr><td>What should be the main properties of voltage sensitive dyes?</td><td>VSD imaging in acute slices
• Fluorescent indicator
• Water soluble
• Fast kinetics
• Linear response
• Minimal capacitive load
• Should not be fluorescent outside the cell
• Fluorescence needs to increase due to depolarization
• Signal as high as possible
• Low phototoxicity

• Dyes: JPW1114(Di-2-ANEPEQ) - Signal is low, very high phototoxicity; labels golgi, ER, nucleus, every organelle
    ○ But only one structure reports fluorescence change -> the plasma membrane</td></tr>
</table>

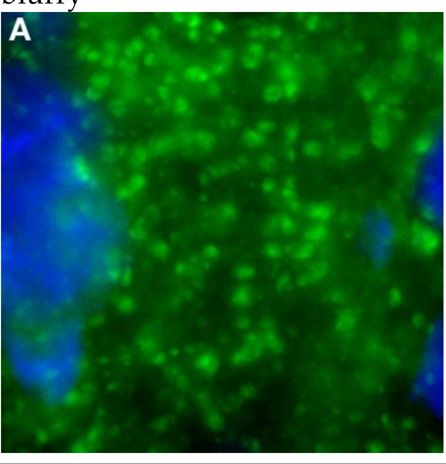
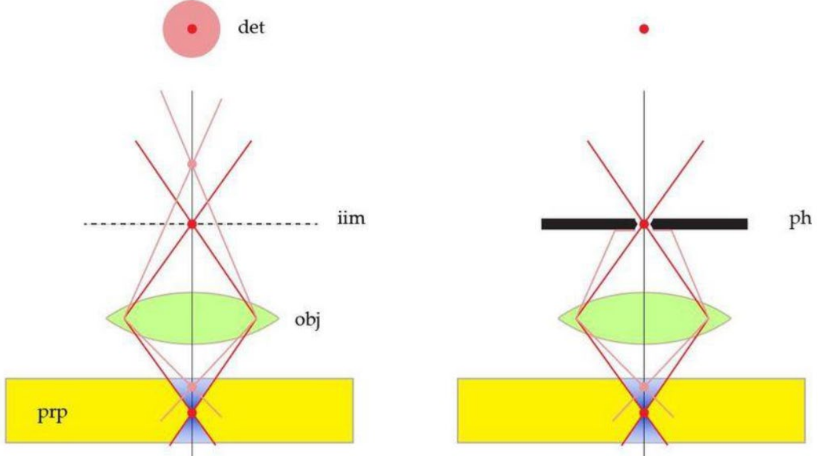


	○ Better suited for smaller cell structures - Better signal to noise ratio (due to surface to noise ratio)
Why voltage sensitive dyes are more appropiate to be used in proximal axon regions?	Data • Proximal axons - Enough fluorescence to check the signal • Peaks are normalized - The action potential should be the same in the same neuron • Variance in speed - Saltatory conduction; fast in nodes of Ranvier,
What causes saltotory conduction?	What causes saltatory conduction (myelin or clustering of channels) • Demyelination in adult neurons with cuprizone • No myelin -> no saltation

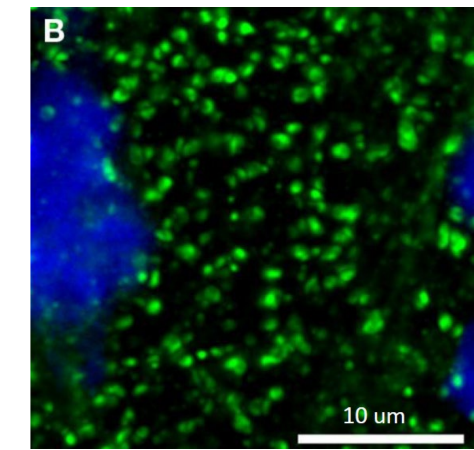
	
Why is the periaxonal space relevant for computational modeling of axons?	What, in the myelin, makes the signal saltatory? • There is a gap between membrane and myelin -> Periaxonal space • When periaxonal data is added to the model, the data matches much better than without the periaxonal space added 
How can voltage sensitive dyes be calibrated?	Problem: Voltage sensitive dyes cannot be calibrated • Solution: • Action potential followed by long hyperpolarization pulse -> serves to calibrate signal

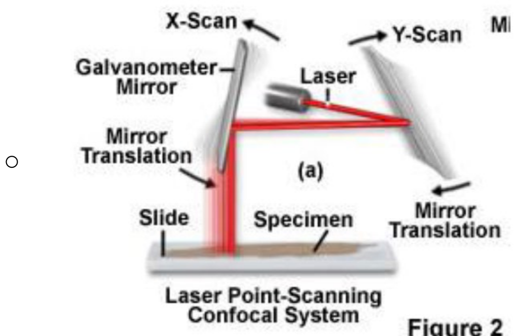
	 *This technique needs a very high frame rate camera* *Long-term imaging is not possible - Very short exposures are required*

Introduction to Fluorescence Microscopy - 1-photon vs 2-photon imaging (Rogier Min)

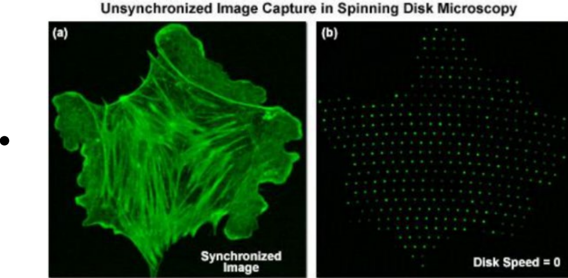
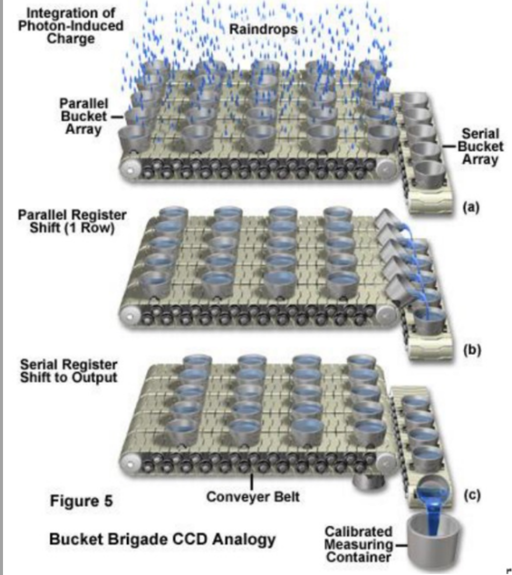
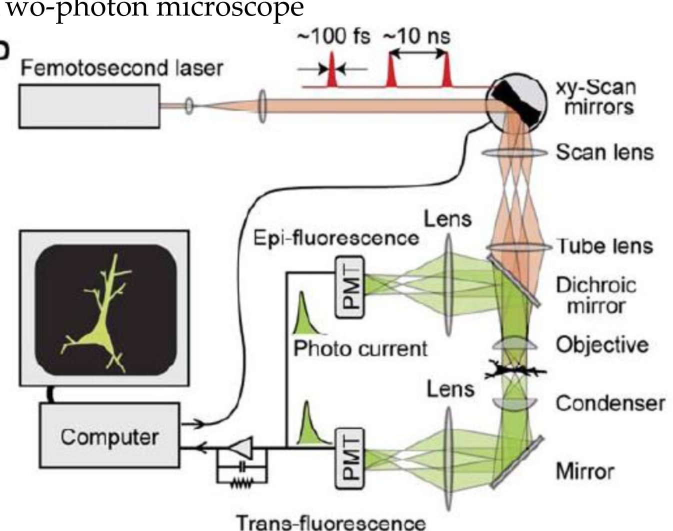
<table>
<tr><td>What are the main problems of widefield microscopy?</td><td>Widefield microscopy main problem - Limited resolution, staining is often required to see sample features
Conventional fluorescence microscopy - High intensity light source needed to activate fluophore; The entire field of view is illuminated
Epifluorescence - Background illumination is quite high, image looks blurry

</td></tr>
<tr><td>What is the principle of confocal microscopy?</td><td>Confocal microscopy - Aimed to overcome problems with widefield microscopy


• Same principle as pinhole camera - Small aperture -> better focus
• Only light that comes from a particular plane of the sample is able to get to the detector - Two points are confocal (same point in the lens)</td></tr>
</table>

<table>
<tr><td></td><td>• 

• In order to change the plane, you change the excitation light or pinhole position</td></tr>
<tr><td>What are the possible ways to move the laser in confocal microscopy?</td><td>Confocal laser scanning microscopy - XY movement in the sample
• Stage-scanning - Stationary laser beam and moving stage platform - Easiest design to achieve
• Beam-scanning - Moving laser beam and stationary platform
    ○ Produced by a galvanometer (rotation movement of mirror changes direction of light)
    ○ 

• Acousto-optic deflector: can produce fast (microsecond) jumps of the laser beam -> more accurate than galvanometer
• Piezo driven objective scanner - Z movement
    ○ Alternative: Electrically Tunable Lens to get z-movement</td></tr>
<tr><td>Describe how Nipkow spinning disk works.</td><td>Nipkow spinning disk microscopy
• Stationary laser and platform, but scan with a disk array of miniature apertures
• Multibeam scanning methods
• Synchronised, rapidly-rotating pinhole discs</td></tr>
</table>

	• 
What are the ways to detect photons in confocal microscopy?	Detection of photons • Oculars • CCD camera (charge-couple device) - Grid of light sensitive pixels (photon intensity are converted into electricity)  • CMOS camera • Photonmultiplier tubes - Extremely sensitive detector, single photon resolution, requires high voltage (1000 V) -> good for low light detector, but no spatial resolution
What is the principle behind two-photon microscopy? How does it compare to confocal microscopy?	Two-photon microscope  • Femtosecond pulse laser - pulse of 100 fs and 10 ns apart

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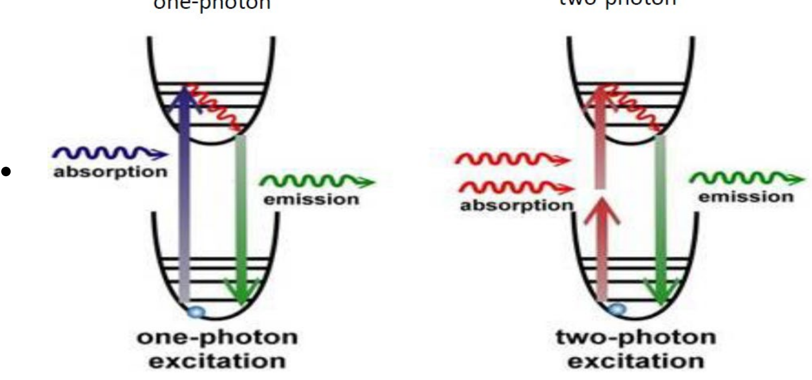
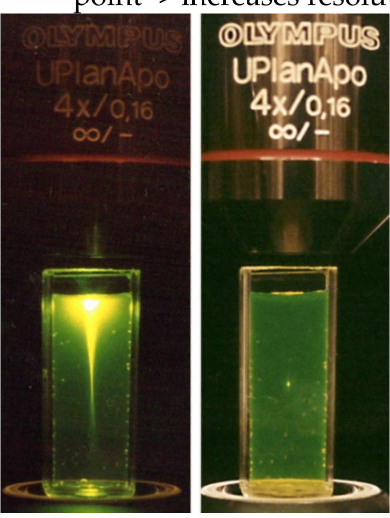
<tr><td></td><td>

- No need for a pinhole - You can look at all the light that comes back

- Main difference to a one-photon microscope - Use two photon with half the wavelength, but super quickly and really focused on one point in the sample, are used to excite the fluorophore
- 

  - Changes of two-photon absorption is maximized in your focal point -> increases resolution compared to widefield



</td></tr>
<tr><td>What are the advantages of 2-photon microscopy?</td><td>

Advantages of 2-photon microscopy

Less scattering, it allows to visualize much deeper in the tissue

Sample is excited only at focal point - Less photobleaching/phototoxicity

</td></tr>
<tr><td>What are the disadvantages of 2-photon microscopy?</td><td>

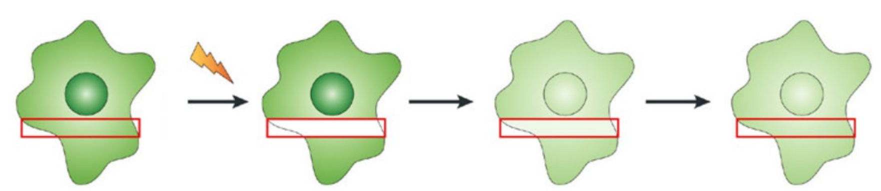
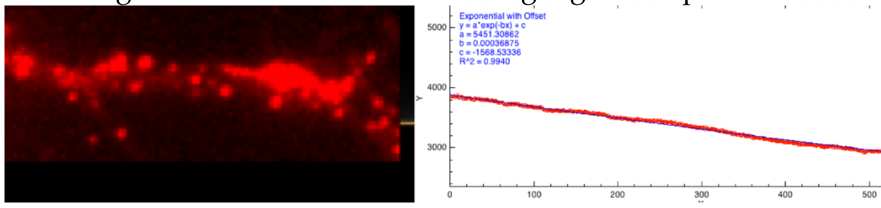
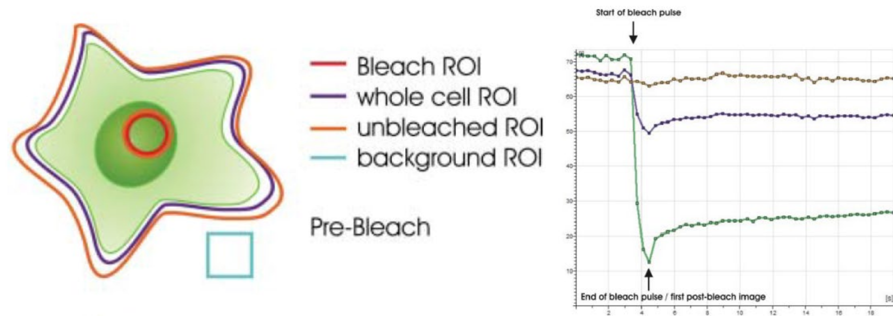
Disadvantages of 2-photon microscopy

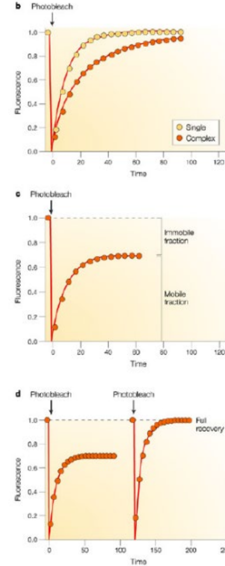
- Expensive
- Worse xy resolution compared to 1 photon
- Not all dyes/fluorophores work well for 2-photon excitation

</td></tr>
</table>

Fluorescence recovery after photobleaching (FRAP)

What is FRAP?	What is FRAP? • Uses localized bleaching of fluorescence to study diffusion kinetics and transport
What are the two different types of FRAP?	Types of FRAP • Confocal scanning microscope - Imaging and bleaching light path are the same • Widefield setup with separate scanning optics - Imaging and bleaching light path are different, setup is more complicated
What does the AOTF (acousto-optical tunable filter) do in the FRAP setup?	Light path for FRAP • Laser light source with AOTF attenuation - Low intensity for imaging and high intensity for bleaching in the same setup • Scanning optics (XY scanning mirrors) -> Moved by galvanometer
What is the basic principle of FRAP?	Basic experimental setup

	 • Sample with fluorescently labelled protein or strucutre ○ Stable and sufficient expression levels of native protein • Determine areas to bleach - Whole cell or particular organelles/ structures • Image baseline time series ○ Make sure the bleaching rate is slower during normal imaging compared to bleaching periods • Bleach the selected areas ○ Area needs to be large enough to measure diffusion ○ Bleaching should be 5x faster than diffusion rate • Image recovery time ○ Image acquisition needs to be fast enough to quantify kinetics
Which control experiments should you do before using FRAP?	Important control experiments • Bleaching rate should be minimal during regular acquisition  • Diffusion of unbound protein - maximum rate of diffusion in the system • FRAP on fixed sample - No recovery is possible
Which ROI measurements can you take from FRAP? What information do they tell you?	Example measurements  • Bleach ROI - Show the recovery • Unbleached ROI (whole cell minus bleach ROI) - Correcting acquisition bleaching • Background ROI - Determines non-specific fluorescence

<table>
<tr>
<td>What are the data processing steps of FRAP?</td>
<td>Data processing
Background subtraction - Removes non-specific signal from the image
Acquisition bleach correction - Removes bleaching due to image acquisition
Fluorescence normalization - Axelrod (recovery rate - how quickly it goes to a new steady state); Siggia (define which fraction of you protein is mobile or immobile)</td>
</tr>
<tr>
<td>How can you tell mobile/immobile fraction of proteins based on FRAP fluorescence?</td>
<td>Parameters


Mobile (Mf) and Immobile (1 - Mf) fraction
The part that does not recover -> immobile fraction (bleached proteins that stay in the same place)
If the protein is not bound to anything (free cytosolic) - it will not have a immobile fraction
Recovery rate (T) - Speed at which molecules diffuse
The faster the recovery, the sharper the curve shape
Recovery half-time - Time required for 50% of total recovery</td>
</tr>
<tr>
<td>What are some biological applications of FRAP?</td>
<td>Examples from experiments
Sharing of vesicles between synapses
FM dyes - Used to study endocytosis
Bleach one synapse - If it recovers fluorescence, then vesicles are shared between synapses in the same neuron</td>
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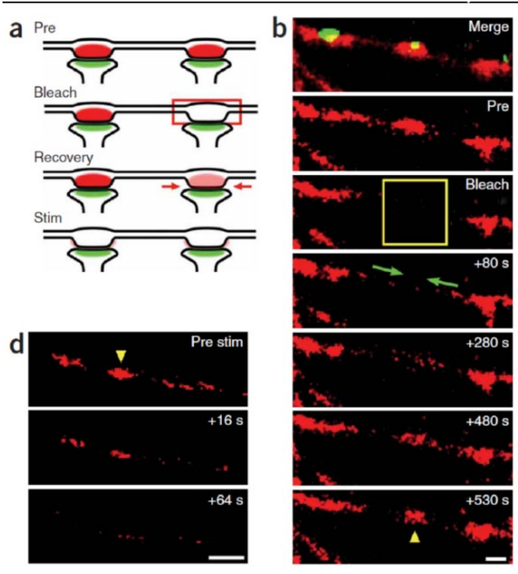
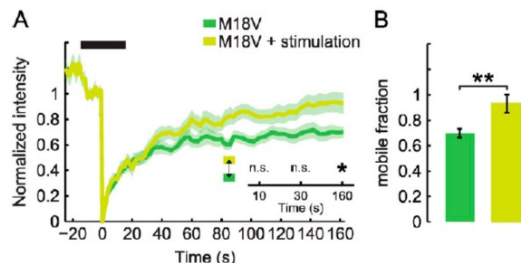
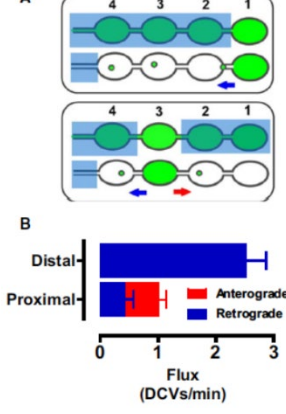


- Mobility of synaptic proteins - Are synaptic proteins recruited during stimulation
    - Fluorescence recovers faster when the neuron is stimulated



- Distributing dense core vesicle
    - Simultaneous photobleaching and imaging - allows for the visualization of individual vesicles
    - DCV accumulate in distal boutons
    - Vesicles are continously distributed bidirectionally



What are the main limitations of FRAP?

Limitations of FRAP

- Bleached proteins cannot be tracked (alternative: photoconvertible proteins)

	• Diffusion during bleaching cannot be measured (alternative: make bleaching as fast and intense as possible) • Measurements are in 2D, but dynamics are in 3D (alternative: acquire 3D volume via z-stacks, difficult at high speeds of acquisition) ○ Less of a problem in axons, big problem in somas

Optogenetics: Things I wish I knew before beginning my PhD (Madison Carr)

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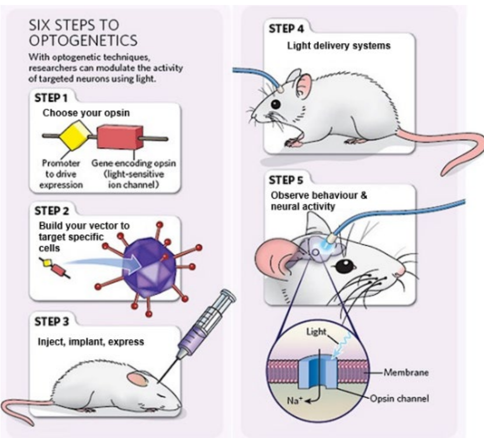
Learning objectives:

1.**Understand the basic principles and applications of optogenetics in slice or behavioral preparations**



2.**Describe the advantages and limitations of applying optogenetic technique instead of other neural manipulations**

3.**You can make informed choices about design and interpretation of optogenetic experiments in relation to:**

o Opsin or "actuator"

o Cellular or sub-cellular compartment targeting

o Light delivery systems

o Neurophysiological read-outs

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What are the differences in resolution for human and animal brain research?

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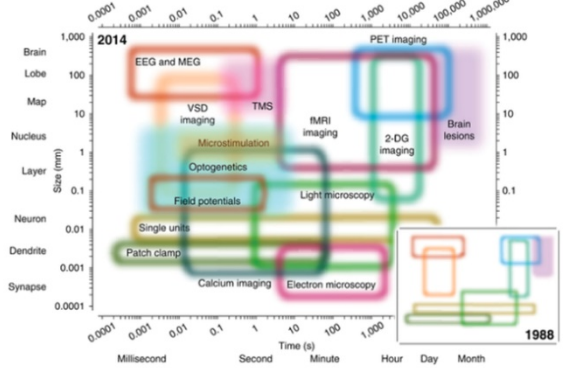
Investigating brain mechanisms



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	• Human research - Correlative, interventions are often clinical, need to be non-invasive • Animal research - Causal, neuronal or subneuronal scale

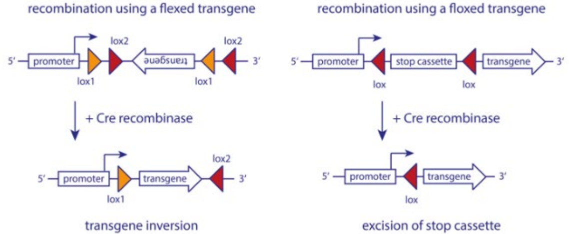
How does one design a functional experiment relation behavior to brain activity?	**Functional experimental design**
	• Observe phenomenon - Hypothesize correlation between behavior and potential mechanism; Obsevations can assess
	    ○ electrical activity (whole cell recordings, extracellular electrodes);
	    ○ calcium activity (photometry/2-photon);
	    ○ activity markers (c-fos, immediately early genes)

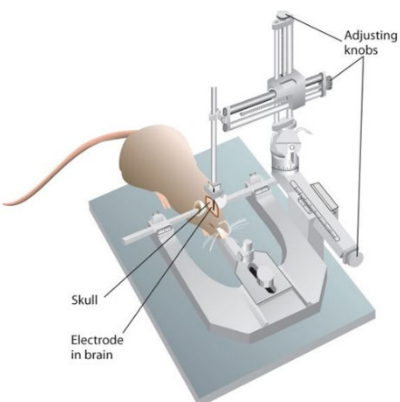
How to assess the relationship between brain activity and behavior?	How to determine the relationship between brain activity and behavior?
	○ Loss of function - It the brain region necessary for behavior?
	    • Lesion studies, pharmacology, genetic knockouts
	○ Gain of function - Can it trigger or induce the behavior? *Is it sufficient?*
	    • Electrical stimulation, pharmacology

What are the main problem of classical interventions in behavioral neuroscience?	Problems with classical interventions • Targeting specifity • Reversibility - Compensatory effects in lesion studies • Temporal resolution
How can optogenetics be used to assess both loss and gain of function?	Optogenetic experiment • Loss of function - Halorhodopsin (assess if the neurons are necessary) • Gain of function - Channelrhodopsin (assess if the neurons are sufficient)

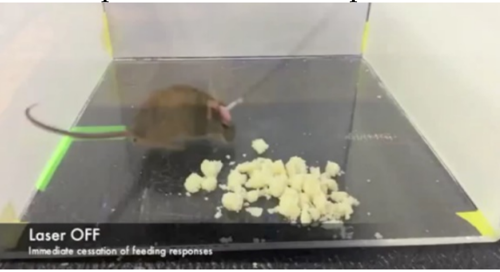
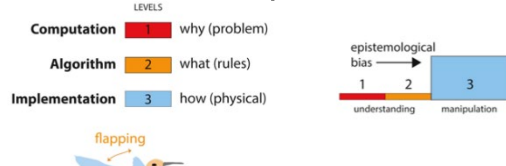
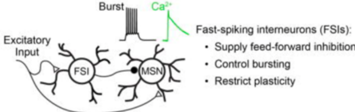
What are practical considerations for choosing your opsin?	Choosing your opsin
	• Actuators - Channels (channelrhodopsin), pump (halorhodopsin/archeorhodopsin), GPCR-associated
	    ○ Pumps are energetically costly - Needs more light and may disturb cellular processes
	    ○ Channels are less costly, need less light
	    ○ Archeorhodopsin - If you expose light for a long time **at the terminals**, it excites the neuron instead of inhibiting it
	• Consider spectral properties, especially if you are combining opto with calcium imaging

	• Step-function opsin - One light pulse opens the channel and it is opened until light at a different wavelength is shined upon ○ Solves light toxicity effects • Consider kinetics - Your opsin need to have the temporal resolution adequate to the mechanism you are studying

What are the different types of transgenes that can be used in a cre system?	Building your vector
	• Cell-type - Promoter-dependent
	    ○ Cre-recombinase is only expressed in neurons of interest - This protein is necessary to flip the transgene or the removal of a stop cassette
	
	
	    ○ Dual-virus approach to introduce cre - Can target specific projection (inject cre-recombinase in one brain regions and viral construct in the other)
	        • *CaMKII - Glutamatergic neurons*
	        • *Remember that neurons with the same cell type project to different neuronal regions*
	• Compartment - Membrane binding domain motifs; suitable for more specific questions

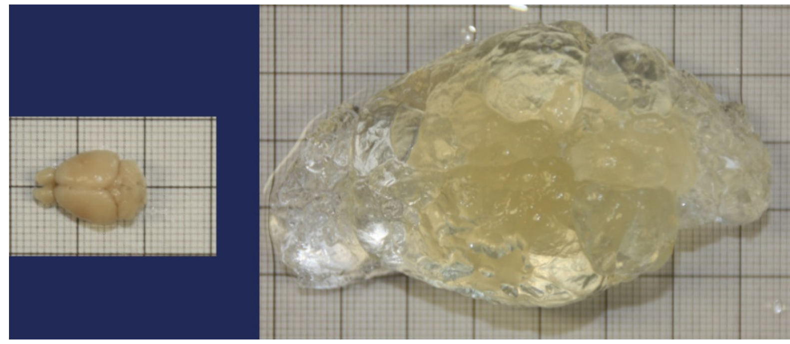
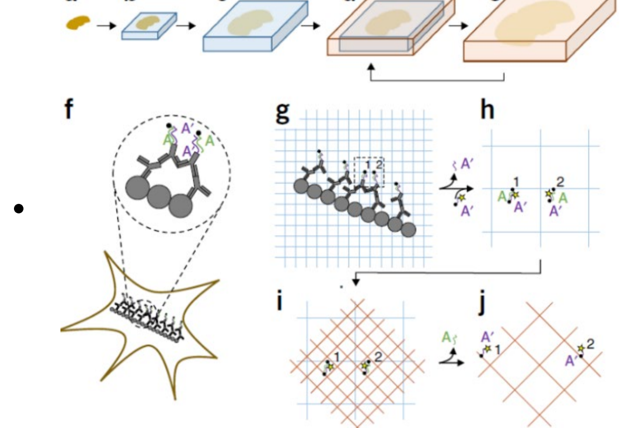
What is the main consideration when choosing a brain region to study relating to the surgery damage?	Stereotaxic surgery  • Centered on bregma • Deep brain regions have different refractive indexes to cortical region
What are practical considerations for a light delivery system?	Light delivery system • Slice or in vivo • Frequency, intensity, pulse width

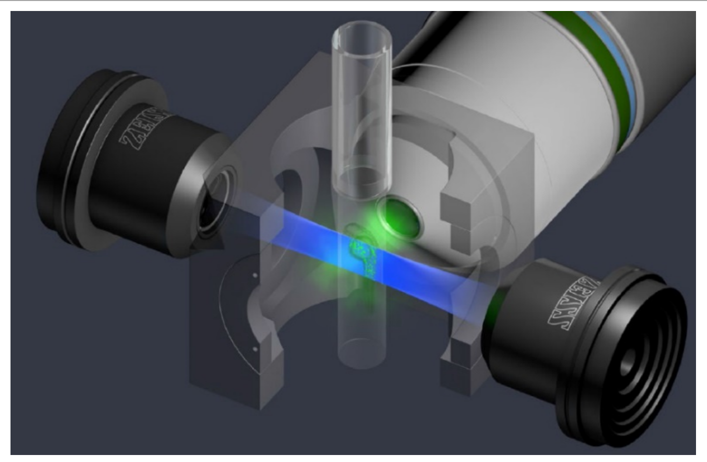
What are practical considerations for behavioral observation in rodents?	Behavioral observation
	• Complex behaviors are controlled by heterogeneous populations
	  ○ Optogenetic identification - What neurons are doing during the behavior?
	  ○ Sham activation - Make sure that the light is not distracting to the performance of the behavior

Why optogenetics experiments don't necessarily point to causality between neuronal activation and behavior?	Limitations of optogenetics • Not necessarily causal - If the animal performs a behavior if the brain region is activated (ecological limitations of behavioral experiments, down/upstream effects to other brain regions)  ○ Many brain regions are redundant - Loss/gain of function does not imply causality
What is Marr's 3 levels of analysis? (not in exam)	Marr's 3 levels of analysis  • Why? • What? • How?
Solve the following exercise:	**Exercise** You want to investigate the whether layer 5 pyramidal neurons (from the prefrontal cortex [PFC]) which are known to project to the dorsal striatum (DS) influence motor 'tic behaviors', which are thought to be caused by excessive MSN firing/output. However, these pyramidal neurons are known to heterogeneously project onto both fast-spiking interneurons and also directly onto inhibitory MSNs. What suggestions do you have (using optogenetics and potentially other tools), to test if either population of DS-projecting pyramidal neurons play a role in modulating MSN output, and thus controlling tic-like behaviors?  • PV-Cre mouse can be used to target interneurons • Fast spiking interneuros - Require a fast-kinetics channelrhodopsin • Tic behavior - Combining behavior with motor learning (motor problem or learning problem)

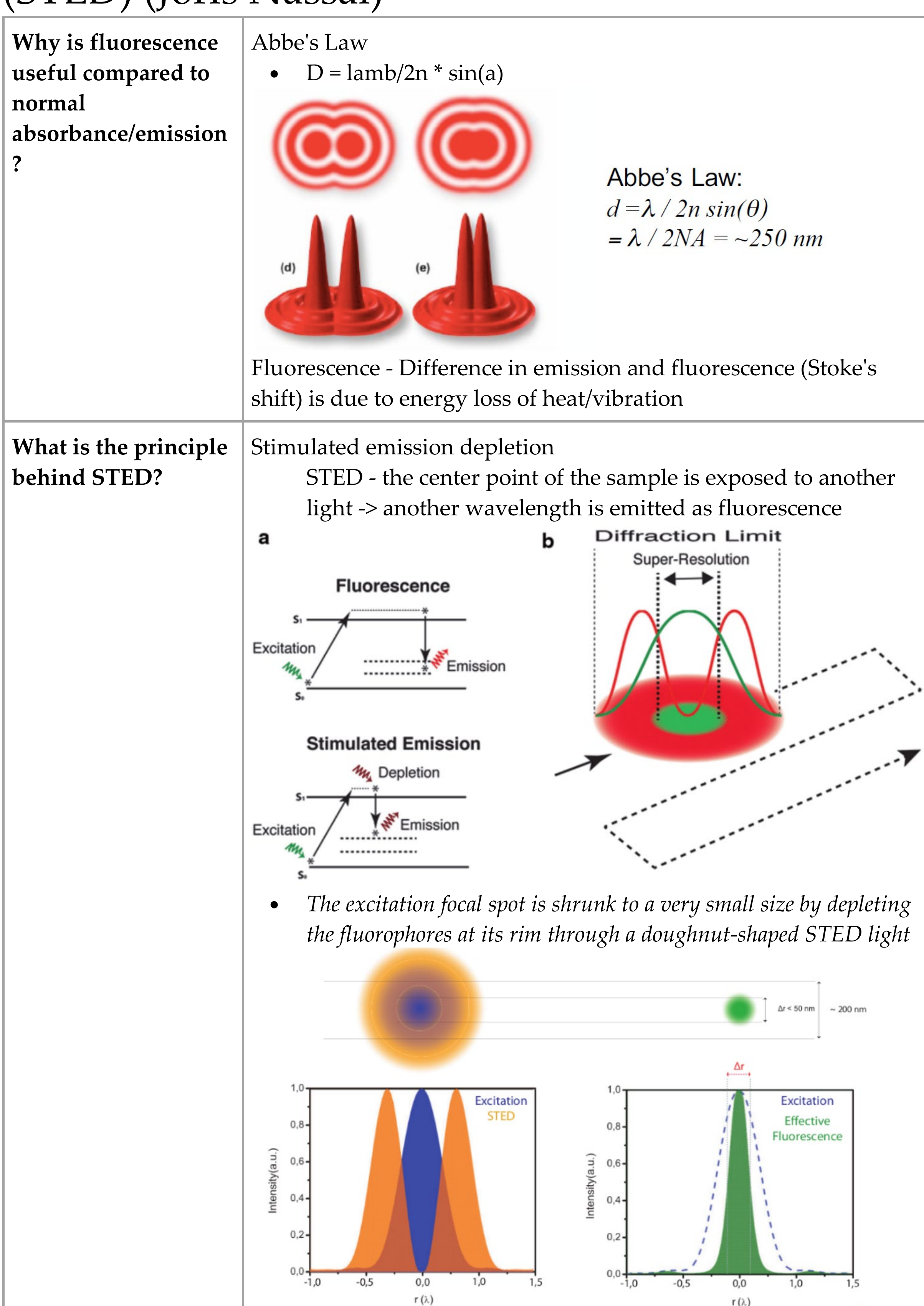
Expansion Microscopy (Bart van Dijk)

	Role of glia in cognition • Mice with subarachnoid hemorrhage have memory problems associated with glia damage (gliosis) • Upon gliosis, astrocytes lose their supportive properties
Why is expansion microscopy potentially more applicable than conventional superresolution techniques?	Imaging astrocytic process • They are very small, smaller than the diffraction limit of light (80 nm) • Superresolution microscopes are really expensive!
What is the principle behind expansion microscopy?	Expansion microscopy • Expend neuronal tissue (90x expansion) - Embedding tissue in polyacrylate gel (same compound used in baby diapers) Fix, antibody stain → AcX >6 h, RT → Gelation 1–2 h, 37 °C → Pro-K Overnight, RT → H_2O 1 h, RT • Proteins need to be denatured • When you add water, the tissue expands - This is not possible for live cells; Tissue also becomes transparent, which is great to reduce light scattering • Expansion is equal in XY, there is some problems in the Z axis due to gravity (1% effect) A B C; RMS Error (µm); Measurement Length (µm); Confocal PSF Size Before ExM Pre-ExM Post-ExM Errors (blue line mean±SD

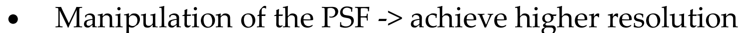
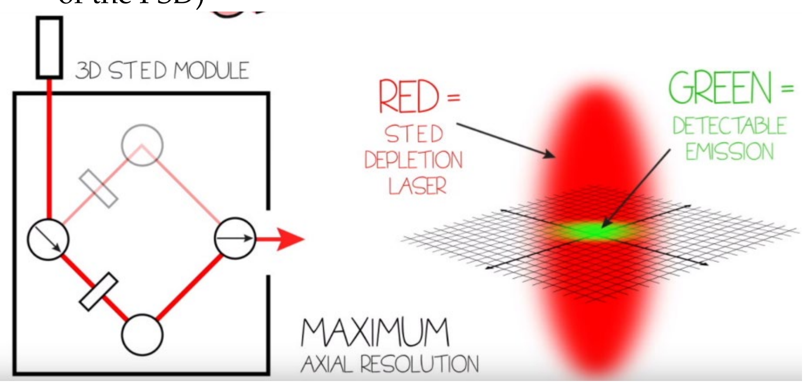
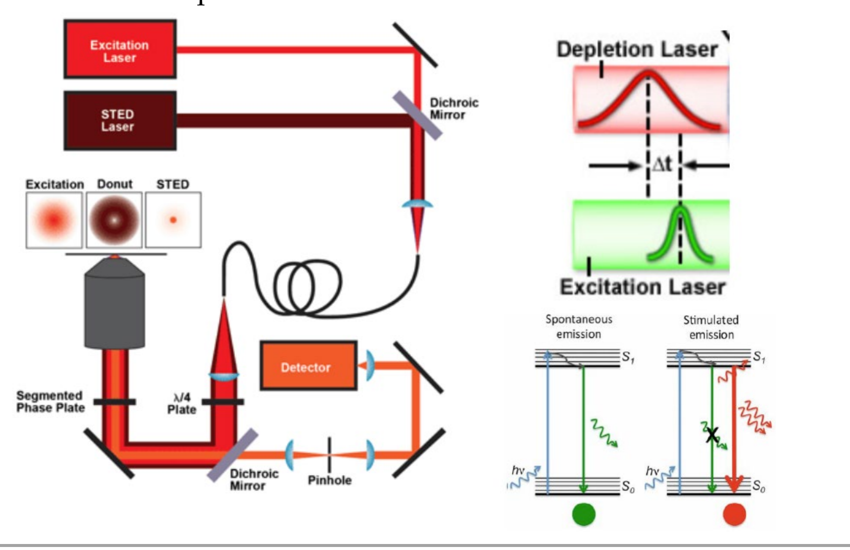
	 **Results**
Which techniques can be used in conjunction to expansion microscopy?	Further expanding tissue • Iterative expansion microscopy (20x in each spatial dimension) - Add new monomers after each step  • Expansion microscopy in combination with light sheet microscopy - 90 degree angle of incidence, reducing *bleaching* of fluorophores (appropriate because tissue is transparent); fast acquisition of data

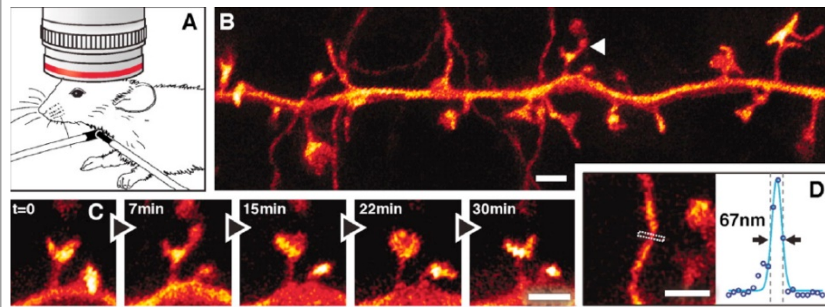
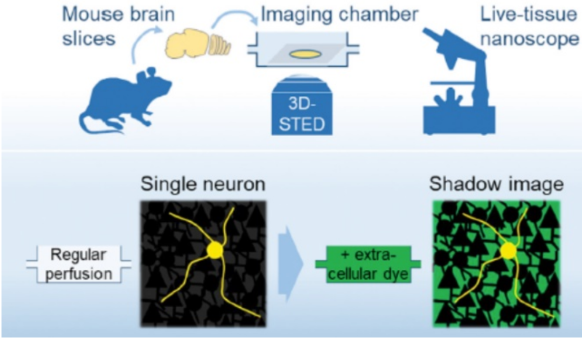
	
What are the advantages of expansion microscopy?	Advantages of expansion microscopy • Superresolution - 60-80 nm • Used with standard lab equipment (confocal) • Can be used for many tissue types • Becomes transparent, better for light microscopy • Can separate multiple protein interaction
What are the disadvantages of expansion microscopy?	Disadvantages • Cannot be used in live cells • Requires some expertise in handling and imaging of gels - For instance, wash away PBS (contains salts) before embedding • Expensive superresolution microscopes still have better resolution

Stimulated Emission Depletion Microscopy (STED) (Joris Nassal)



Why is fluorescence useful compared to normal absorbance/emission ?	Abbe's Law • D = lamb/2n * sin(a) Fluorescence - Difference in emission and fluorescence (Stoke's shift) is due to energy loss of heat/vibration
What is the principle behind STED?	Stimulated emission depletion STED - the center point of the sample is exposed to another light -> another wavelength is emitted as fluorescence • *The excitation focal spot is shrunk to a very small size by depleting the fluorophores at its rim through a doughnut-shaped STED light*

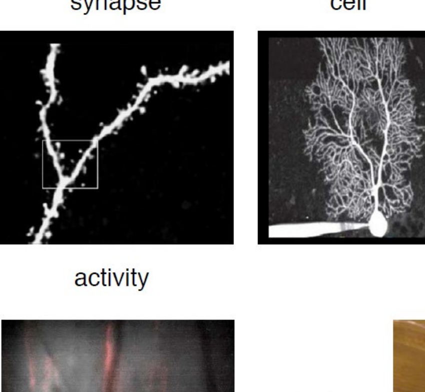
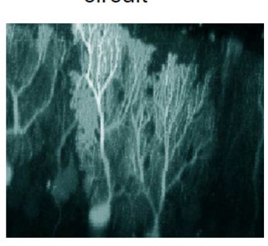
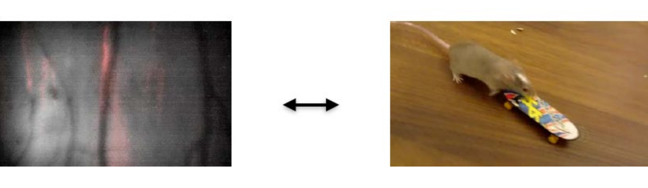
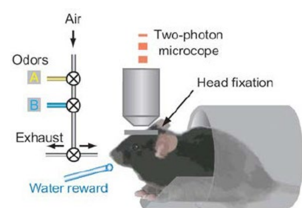
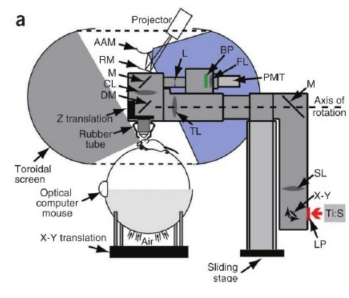
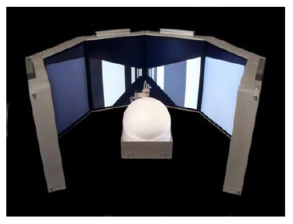
	 • Manipulation of the PSF -> achieve higher resolution • The more intense your depletion laser is, the higher the resolution
How 3D STED controls the shape of the PSF?	STED in 3D (svi.nl) • Depletion at top and bottom of PSF (system of mirrors that control the doughnut depletion laser, controlling the shape of the PSD)  ○ Some fluorescence intensity is lost (only the center of the fluorophore is detected) ○ Z resolution is roughly 100 nm
What is the overall design of a STED microscope?	STED microscope 

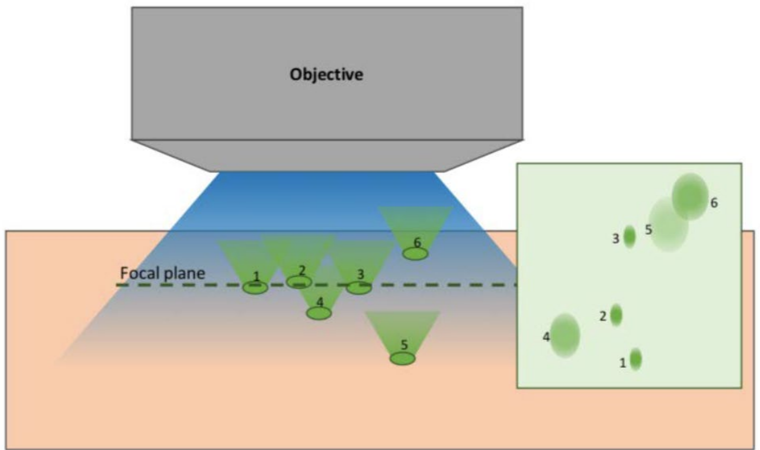
	• Excitation and STED laser need to be very precisely aligned (spatially and temporally, 100s of picosends) • Scanning mirror moves the laser through your sample ○ You need STED lasers for every wavelength of fluorophore, but it two fluorophores are close enough (GFP and YFP) you only need one • Pinhole is present like normal confocal microscopy
Can STED be used in living animals?	In vivo STED imaging • Animal is head fixed 
What are the advantages of STED?	Pros of STED • Higher spatial resolution (XY and Z) • Physical superresolution -> does not require subsequent image reconstruction, like PALM or STORM • Fast scanning is possible • Live cell and in vivo capable - Possible to combine different fluorophores with 2-photon microscopy • Multicolor is possible
What are the disadvantages of STED?	Cons of STED • Very high intensity of depletion laser necessary (phototoxicity) • Fewer photons collected due to smaller effective fluorescent spot -> more sensitive detectors are needed • System needs to be well aligned to obtain super resolution
What is SUSHI?	Superresolution Shadow Imaging (SUSHI) - Combination with 3D-STED 

	• Acute slices • Extracellular dye is added - Unlimited pool of fluorophores -> shadow of the neuron ○ Very precise view of the extracellular space

- STED is patented by LEICA
- STORM is patented by Nikon

Miniaturized Microscopes (Tycho Hoogland)

Why is the miniscope a useful tool in neuroscience?	Imaging can link activity to behavior • Relating the activity of the brain in multiple scales to the final behavior synapse cell circuit   activity behavior 
What are the problems with head-fixed experiments?	Imaging in restrained animals  • Problems - Lacks vestibular inputs, high levels of stress, limited behavioral repertoire, habituation takes a long time
What are the advantages of using a mobile environment with a head-fixed animal?	Imaging in treadmills • Head fixation but the animal is still able to perform locomotor tasks • Combination with VR - Visual inputs, more realistic setting   • Advantages: Can use tabletop microscopes, well-constrained behavior, pharmacology and whole-cell recordings are easier • Same disadvantages as before
What is GCaMP?	GECI can register single action potentials

	• Green Calmodulin Protein (GCaMP) - GFP + Calmodulin + M13 • Non-linear relationship between fluorescence and calcium concentration
What are the advantages and disadvantages of using viral injections instead of transgenic lines?	Delivery of GECI using viral vectors • Virus (AAV) that allow transduction in cells of interest • Promoters can specify neuronal population • Possible problem - Overexpression of GCaMP • Alternative: Transgenic line (lower expresssion)
What is the main problem of widefield imaging?	Widefield imaging - Collection of light from different focal planes 
What is the miniscope and its main applications?	Miniscope - Minituarized fluorescence microscope, sufficiently small such that it can be carried on the head of an animal • Reduced stress compared to head-fixed setups • Intact vestibular system • Suited for sleep studies, social interaction, action sequencing, fidgeting, vocalization (learning of stereotypical sounds), bird flight
How do two-photon miniscopes work?	Two photon miniscopes • Requires a high frequency pulse laser (100k euros) • Requires efficient optical fibers (femtosecond transmisison of information), miniturized scanning mechanism, small objective lens, effective fluorescence collection

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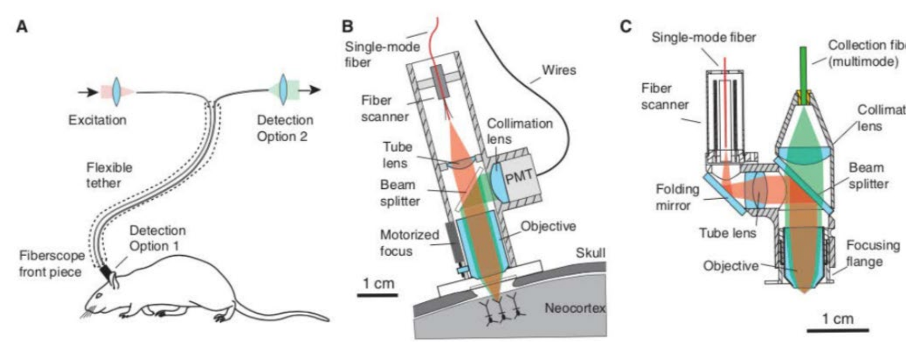

- PMT - Photon multiplier tube
- Most tabletop two-photon systems have the detector really close to the sample
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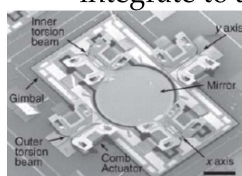
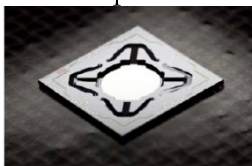
<td>What are the two types of scanning mechanisms used in two-photon microscopy? Which one is more appropriate for the miniscope?</td>
<td>
Miniturized scanning mechanisms

- Piezoelectric bender for fiber scanning - Not very stable
- Microelectrical mechanical scanner - More stable, easier to integrate to a miniscope

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<td>What are the main learning points from the history of 2-photon microscopy?</td>
<td>
History 2-photon miniscope

- Helmchen (2001) - 25 g 2P miniscope
- Sawinski (2009) - 5.5 g 2P miniscope - Too heavy for mice, but suitable for rats
- Piyawattanametha (2009) - 2.9 g - 2 P MEMS device for scanning
- Zong (2017) - 2.2 g - 2 P - Flexible optic fiber bundles, higher collection efficiency and allows the animal to move around more freely
- Ozbay(2018) - 2.5 g - One fiber for collection and excitation, lower numerical aperture

- Considerations: MEMS is better suited for miniscope, high quality objective, high efficient and flexible fibers
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<td>What are the possible configurations of the miniscope?</td>
<td>
History 1-photon miniscope

- Flusberg (2008) - 1.1 g, Good enough signal
- Ghosh et all (2011) - Simplified fluorescen microscope, CMOS sensor, LED excitation; Open source
    - Inscopix - 100.000 dollars
    - UCLA miniscope - Less than 1500 dollars

  1 photon miniscope configurations
    - Superficial imaging - GRIN (Gradient refractive index lenses) objective touches the brain
</td>
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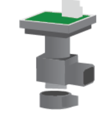
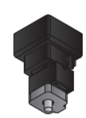
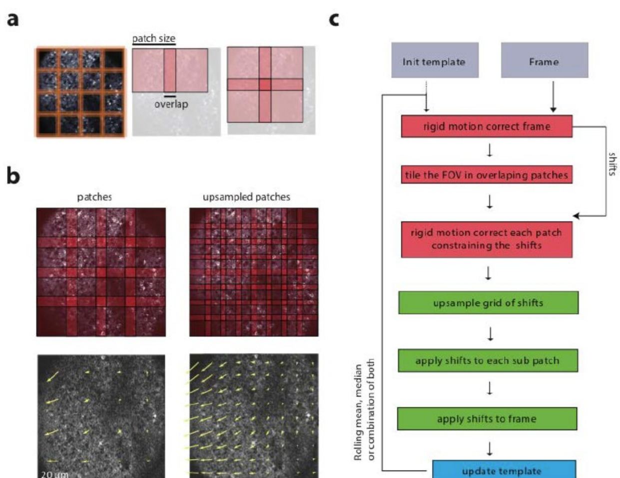
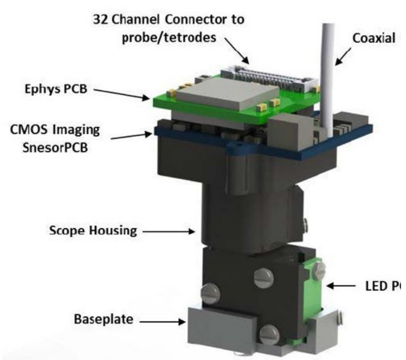
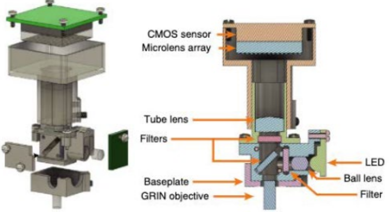
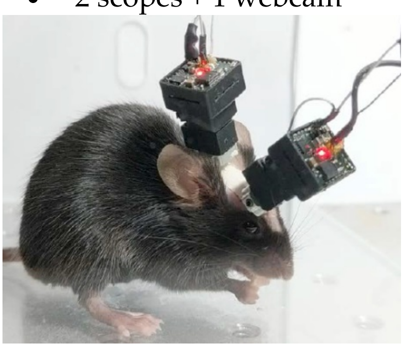
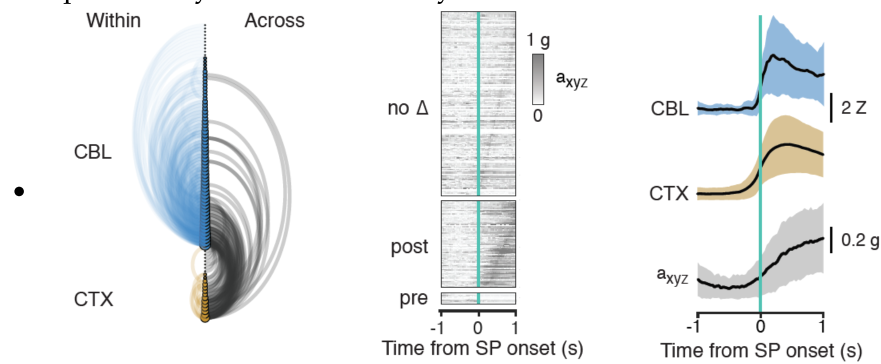
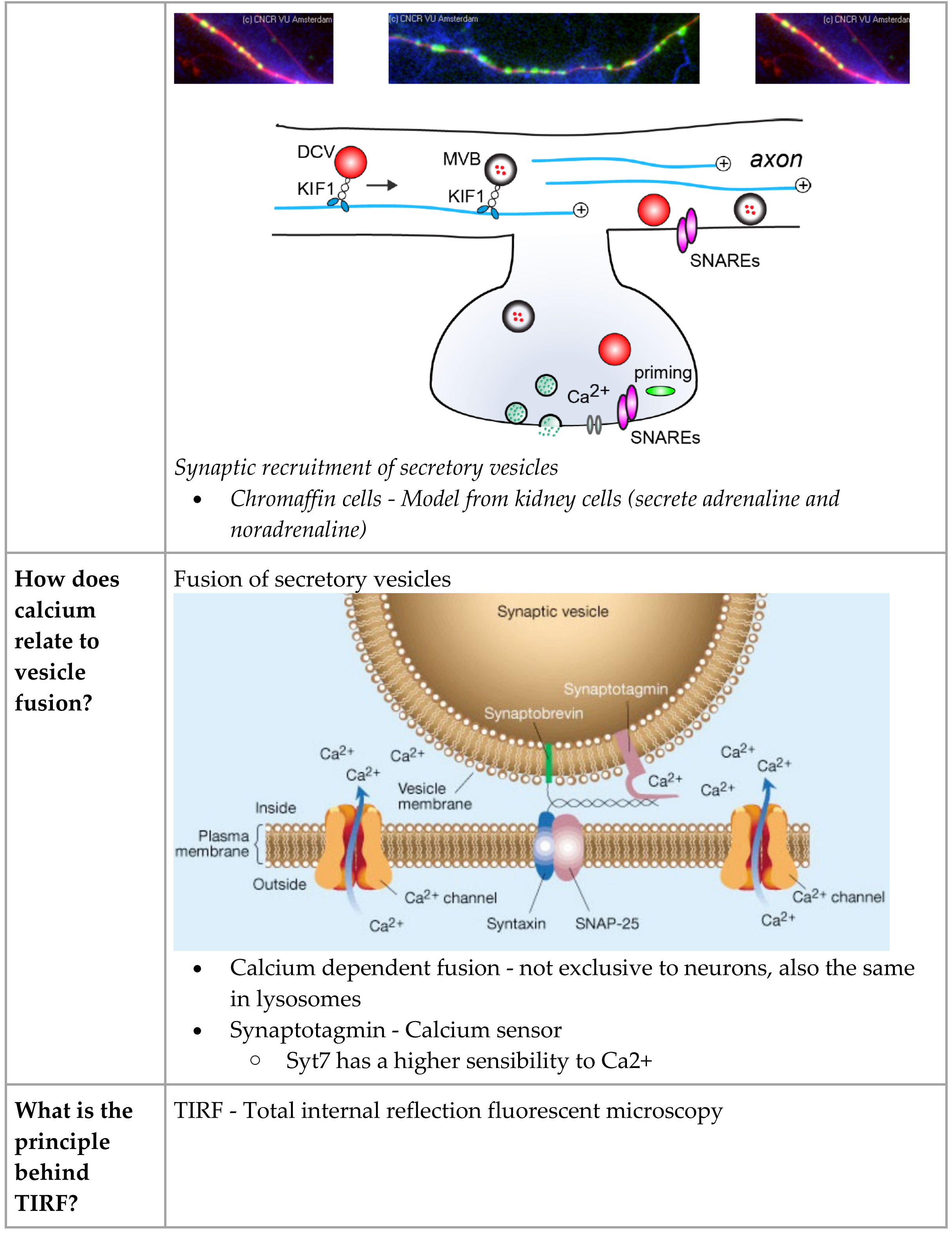
	• Deep imaging - Relay (long lens) is inserted into the brain
What are the currently available miniscopes?	Review - Aharoni & Hoogland FinchScope Dim: 10 x 6 x 21 mm Wired: 1.8 gram Wireless: ~ 4 gram FOV: 880 x 600 µm Frame Rate: 30 Hz Focus: turret DAQ: Arduino Software: MacOS  miniScope Dim: 12 x 12 x 20 mm Wired: 2.4 gram FOV: 1.1 x 1.1 mm Frame Rate: 10 Hz Focus: turret DAQ: Opal Kelly Software: Win & Mac UCLA Miniscope Dim: 16.5 x 13 x 22.5 mm Wired: ~ 3 gram Wire-free: 4.5 gram FOV: 700 x 450 µm Frame Rate: 60 Hz Focus: linear slider DAQ: custom PCB Software: Win CHEndoscope Dim: 15.9 x 17 x 32.5 mm Wired: 4.5 gram FOV: ~ 500 µm across Frame Rate: 20 Hz Focus: turret DAQ: direct to PC Software: Win & Linux  NINscope Dim: 11 x 11 x 18 mm Wired: 1.6 gram FOV: 00 µm Frame Rate: 30-120 Hz Focus: linear slider DAQ: direct to PC Software: Mac, Win & L Built-in: G-sensor, optc • CHEndoscope - everything is 3D printed, easier to implement

Define some practical considerations when acquiring/processing data from the miniscope.	Practical considerations
	• Interface
	  ○ DAQ hardware - Integrates via USB3.0 (it is better to use a laptop instead of tower PC, mobility)
	• D-mannitol - increases extracellular space, increase spread of virus
	  Also decrease brain pressure during
	Motion-correction
	
	
	  • NoRMCorre - Python and MATLAB toolboxes
	  • Non-linear movement corrections - the brain is shearing (because it plastic), linear movement correction do not work
	Extracting neurons and signals using CNMF-E
	  • Extraction of component (individual neurons) from the raw data

	 • Option - MIN1PIPE - deep neural network deconvolution
What are the new types of miniscopes?	**New miniscopes** UCLA miniscope (wireless) • Good for unrestrained behavior over larger distances • Social interaction • 15 minutes of recordings in a SD cards • Battery adds some weight Electronphysiology miniscope  • 32-64 channel recording Miniscope for volumetric imaging  • Computationally define where the light is coming from the signal intensity
What are the main advantages of the	NINscope • CMOS sensor - More light collection

NINscope over other miniscopes?	• Optogenetic LED driver • 2 scopes + 1 webcam  • DLP 3D printer - Rapid prototyping • Software was also developed in-house • Can be used two scopes at a time - Behavior is not greatly affected
	Example data: synchronous activity between cerebellum and cortex •  Optogenetic actuation with NINscope Deep brain visualization is also possible • Cells from the right striatum only activate during left turns
What is the future of miniscopes?	Future of miniscopes • Tunable lenses - Define focal depth in software • Dual-color miniscopes - Two fluorescent GECIs could be used • 3D printed optics -> further miniaturization • Lensless system -> Detect surface for the entire cortex

Single vesicle imaging (Ruud Toonen)



	Synaptic recruitment of secretory vesicles • *Chromaffin cells - Model from kidney cells (secrete adrenaline and noradrenaline)*
How does calcium relate to vesicle fusion?	Fusion of secretory vesicles • Calcium dependent fusion - not exclusive to neurons, also the same in lysosomes • Synaptotagmin - Calcium sensor ○ Syt7 has a higher sensibility to Ca2+
What is the principle behind TIRF?	TIRF - Total internal reflection fluorescent microscopy

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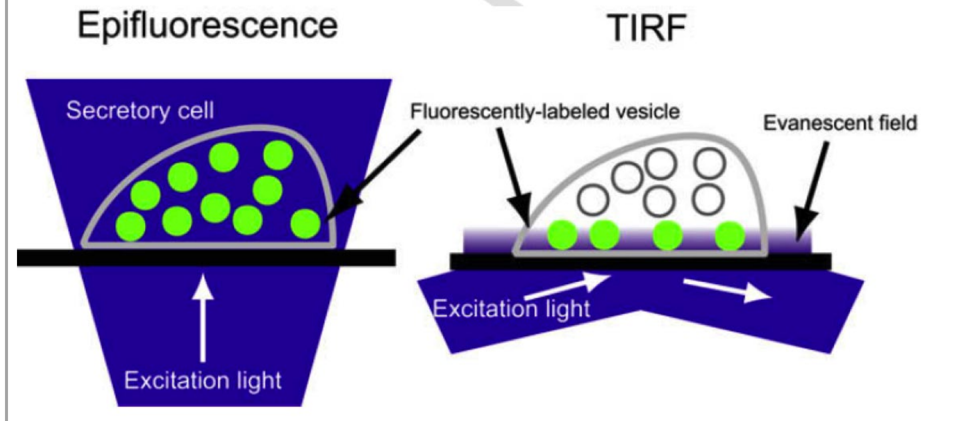
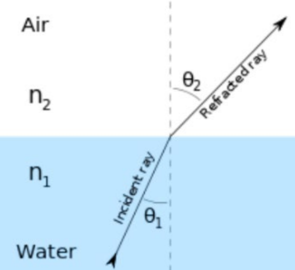


- As opposed to normal epifluorescence microscopy, it only iluminates part of the sample (depends on the angle of excitating light)
- Evanescent field - Exponential decrease of light gradient in the sample
</td>
</tr>
<tr>
<td>What is Snell's Law?</td>
<td>
Snell's Law

- The speed of light depends on the medium it travels through

$n_1 \sin \theta_1 = n_2 \sin \theta_2$

- From high refractive index to low refractive index - Velocity increases, bend away from normal



- Diamonds have the highest refractive index - 2.4
</td>
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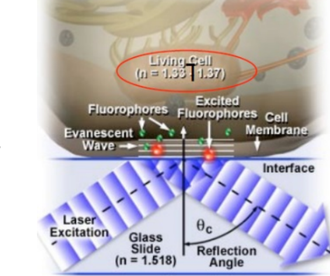
<tr>
<td>What is the critical angle?</td>
<td>
Critical angle - Angle at which total reflection occurs

- Living cells' refractive index depends on the organelle configuration inside it (average = 1.38)
- Glass slip - 1.51

cells

- 

Glass/cell 1.35/1.518 ±63°

  - This is the same principle used in fiber communication
</td>
</tr>
<tr>
<td>What is an evanescent</td>
<td>Evanescent wave</td>
</tr>
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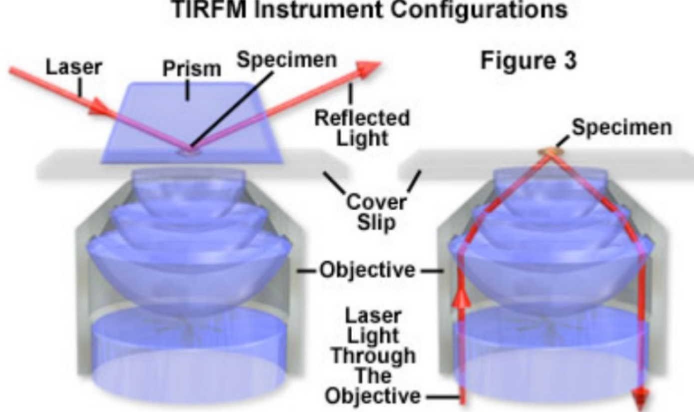
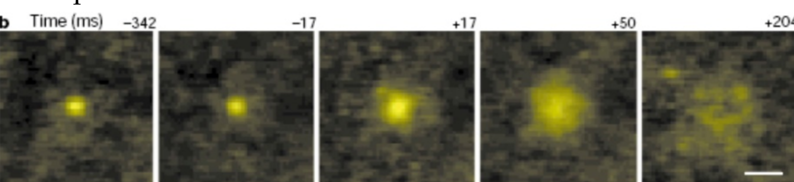
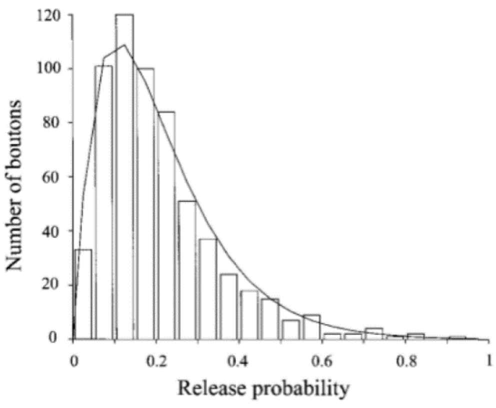
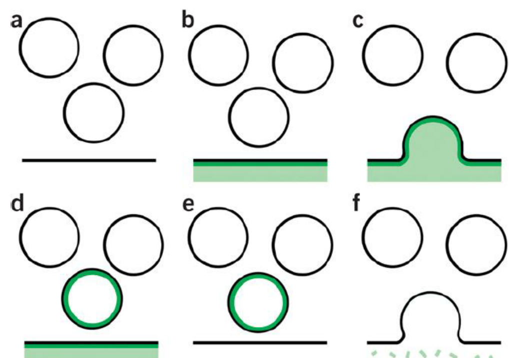
wave? Why is it useful for microscopy?	Evanescent Wave Exponential Intensity Decay
	Figure 2 — Evanescent Wave; Aqueous Buffer Phase (n = 1.33); Glass Phase (n = 1.518); Reflected Collimated Laser Illumination; Penetration Depth (d); Z; I_z
	• Penetratio depth depends on
	  ○ Wavelength - Violet light penetrates more than red light
	  ○ Angle
	  ○ Refractive index difference
	• Remember that membrane is not completely juxtaposed to the glass slip - Fluorescent vesicles in the same relative position to the glass slip might have different distances to the membrane
	• Changes in intensity in Z-axis is in the order of nanometer

How does the resolution of TIRF compare to normal confocal microscopy?	Confocal vs TIRF confocal; TIRFM; (A); (B); 0.5; 0; 0.5 µm • Point spread function on TIRF - XY resolution is the same, Z resolution is ten times better than confocal
What are the two possible TIRF setups?	TIRF setups

	 • Crude - Normal objective, with a prism on top of the cell, shining a laser at the critical angle • Modern - Objective with very high NA, laser light is sent through the objective; Only a small percentage of the objective is used, so a very strong laser needs to be used
What are the advantages of TIRF?	**Advantages of TIRF** • Very high axial resolution • Changes in fluorescent intensity correlate with Z distance from the glass slip • Very high signal to noise ratio and minimal photobleaching - only a few fluorophores are excited at a time • High acquisition rate - 2000 frames a second
Which types of phenomena can be studied with TIRF?	Example  • Goldfish bipolar cells - Neuron with only one axon and one giant synapse (which also makes a synapse on the glass cover slip) • Study concluded there were four active zone (regions in which the vesicles fused) • **Up to this date, there are no other model neurons that work! Only goldfish bipolar cells.**
	Dynamic microtubules regulate dendritic spine morphology • Label of plus ends of microtubules - They infiltrate the dendritic spine ○ Debunked hypothesis that vesicles switched from kinesin to myo5 transport • *Neurons can grow on glass cover slips, but a lot slower than with glia cells* • *If you grow neurons with glia, if it difficult to be sure if you are visualizing neurons of glia*

What is release probability?	**Principles of synaptic transmission**
	• Most neurons in the CNS have a very low release probability
	
	  ○ Varies from synapse to synapse
	  ○ *Electrophysiology provides the sum of all synapses - To analyse an individual synapse you need another technique*
	• Sensory and motor neurons have a very high release probability

How do FM dyes work?	**FM Dyes**  • Only fluoresces when it binds to the membrane • After a while, the synaptic vesicles also become labelled (endocytosis) • You can wash the extracellular FM dye, not the dye inside the vesicles • Taking a picture before and after the wash - Allows for the count of the number of vesicles (FQ - intensity from a single vesicle; quanta)
Why FM dyes can be useful for both light and electron microscopy?	FM dye pictures are photoconvertible • After fixation for EM pictures - FM dye looks darker

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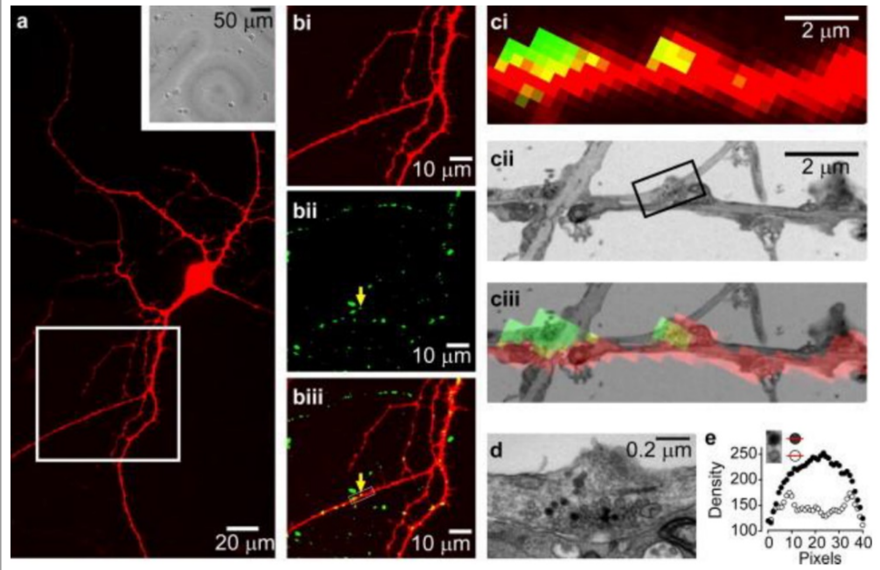
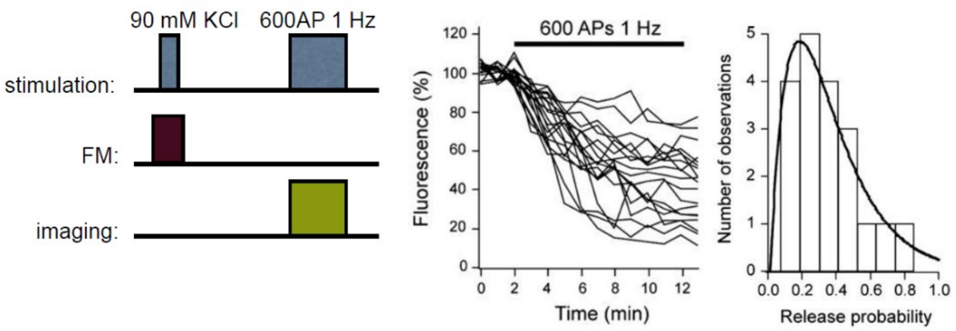
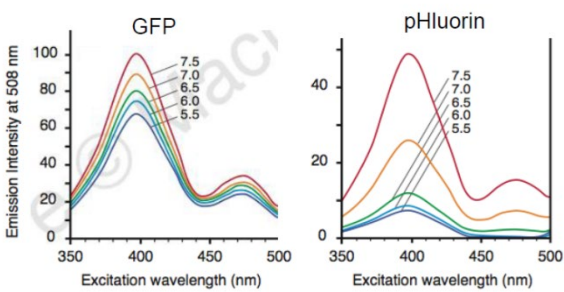
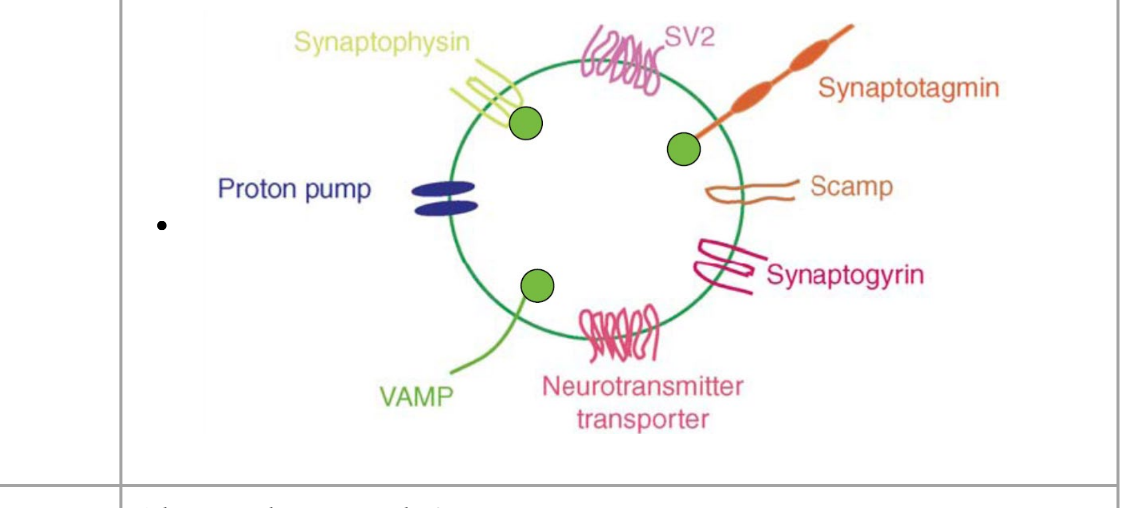
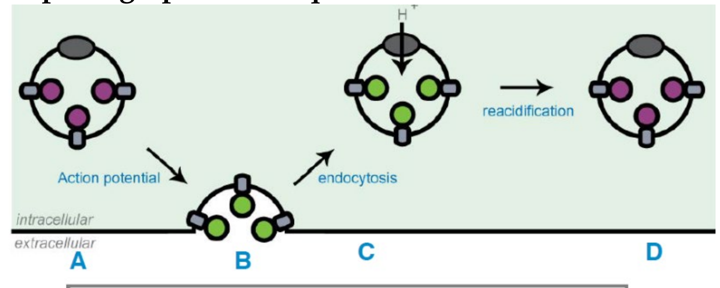
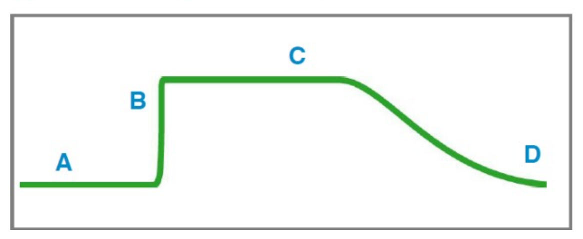
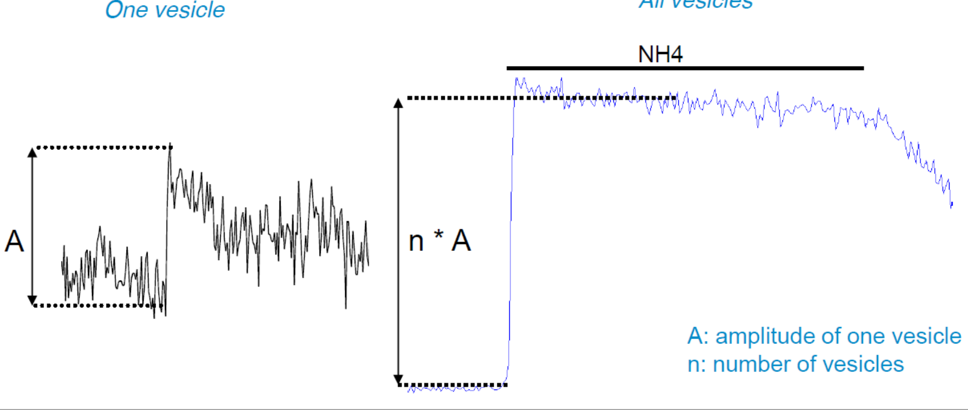
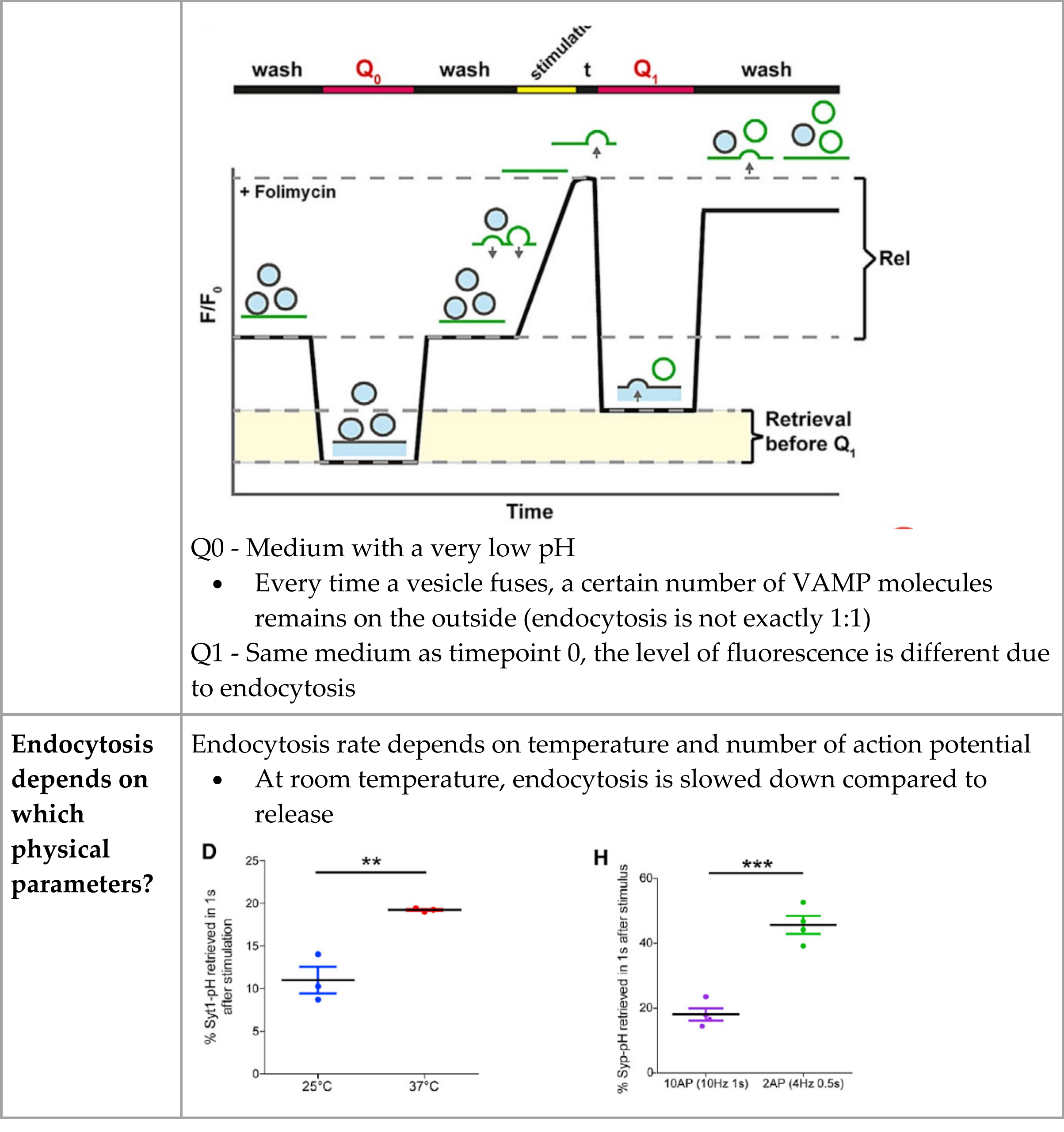
<td>
</td>
</tr>
<tr>
<td>How can you use the concentration of FM dye to measure vesicle release?</td>
<td>Live visualization of vesicle release
• 90 mM KCl - Depletes all readily available pool of vesicles, label all vesicles
• The destaining of FM dye is an indication of vesicle release


• Reason why release probability is higher - To label the vesicles, you had to depolarize the cell, local calcium was temporarily increased</td>
</tr>
<tr>
<td>What is phluorin? For which biological phenomenon can it be used?</td>
<td>pHluorin - pH sensitive variant of GFP


• More acidic medium - More fluorescence
• You can measure the pH inside the organelle from the emitted light - Useful to check maturation in lysosomes
• You can add pHluorin to tag transmembrane proteins in the synaptic vesicles</td>
</tr>
</table>

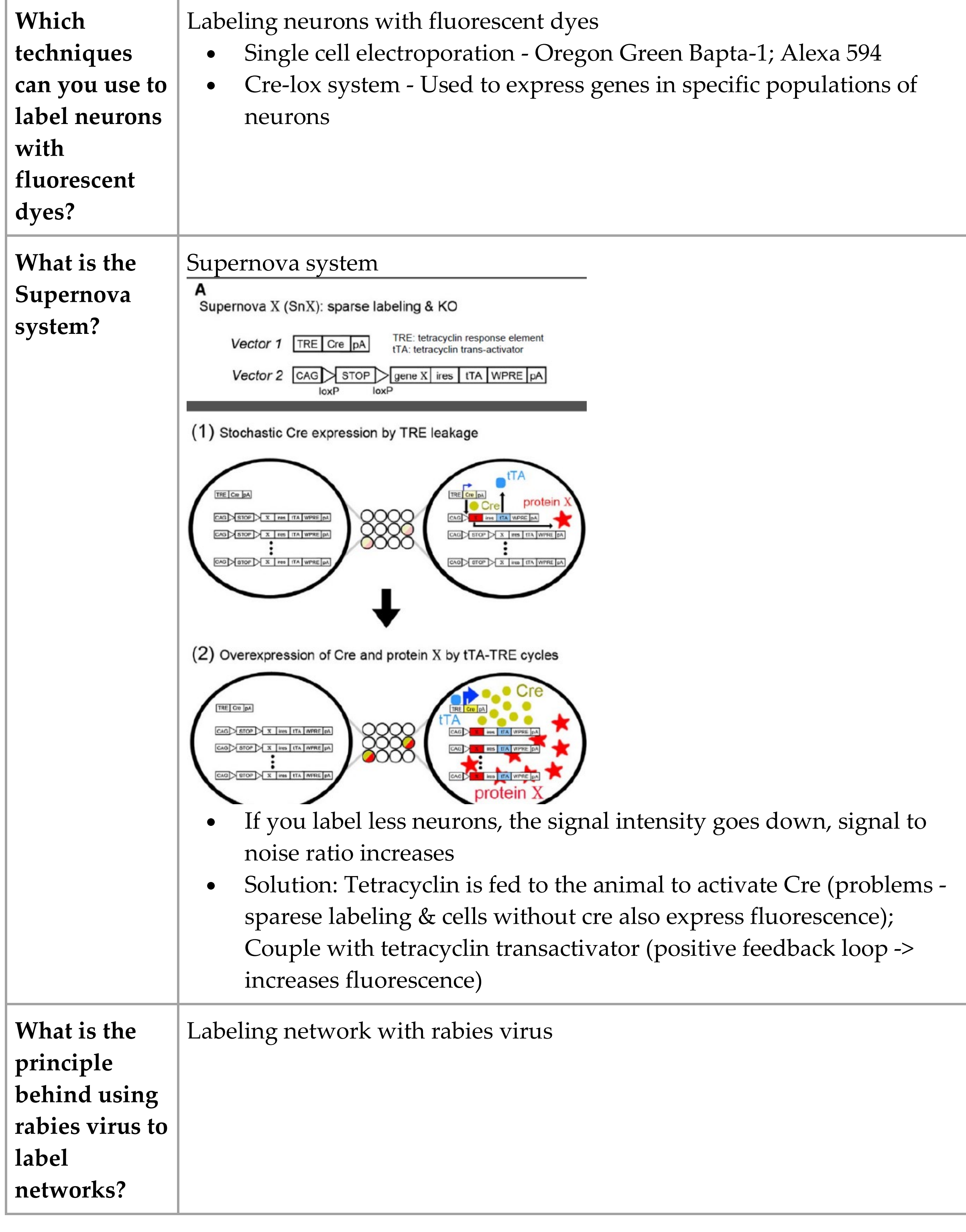
	• 
Why are vesicles acidic?	Why vesicles are acidic? • V-ATPase pump - The hydrogen pumped in is used by neurotransmitter antiporters • The timing is fixed (there are two V-ATPase pumps per vesicle) - Takes around 3 seconds • **Explain graph - Exam question** • 
How can NH4Cl used with phluorin to calculate the number of vesicles in the sample?	NH4Cl - Instantly neutralizes the interior of the synaptic terminal • You can calculate the number of vesicles by knowing the value of one individual quanta 

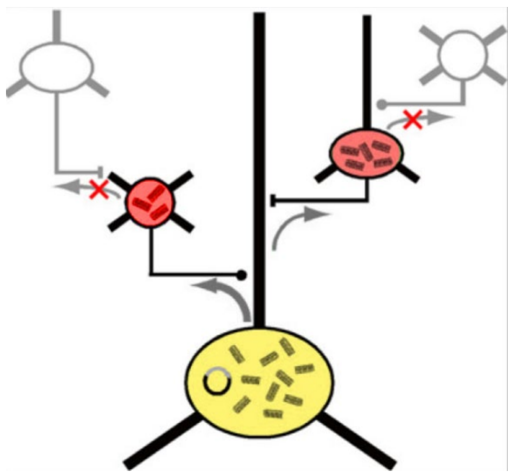
What is bafilomycin?	Bafilomycin blocks reacidification after endocytosis • No hydrogen inside vesicle, no neurotransmitter exchange bafilomycin H+ Action potential endocytosis intracellular extracellular A B C D A B C D
How can bafilomycin and ammonia be used to calculate the poolsize of vesicles?	Bafilomycin + Ammonia - Allows for the calculation of poolsize • 10Hz NH_4Cl FM spH Non-Releasable pool Releasable pool Total vesicle pool 0 1 2 3 time (min)



	Q0 - Medium with a very low pH • Every time a vesicle fuses, a certain number of VAMP molecules remains on the outside (endocytosis is not exactly 1:1) Q1 - Same medium as timepoint 0, the level of fluorescence is different due to endocytosis
Endocytosis depends on which physical parameters?	Endocytosis rate depends on temperature and number of action potential • At room temperature, endocytosis is slowed down compared to release

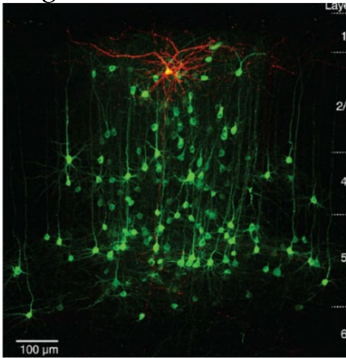
Calcium Imaging I - Visualizing structure and function of developing synapses (Christian Leveelt)

Which techniques can you use to label neurons with fluorescent dyes?	Labeling neurons with fluorescent dyes • Single cell electroporation - Oregon Green Bapta-1; Alexa 594 • Cre-lox system - Used to express genes in specific populations of neurons
What is the Supernova system?	Supernova system  • If you label less neurons, the signal intensity goes down, signal to noise ratio increases • Solution: Tetracyclin is fed to the animal to activate Cre (problems - sparese labeling & cells without cre also express fluorescence); Couple with tetracyclin transactivator (positive feedback loop -> increases fluorescence)
What is the principle behind using rabies virus to label networks?	Labeling network with rabies virus

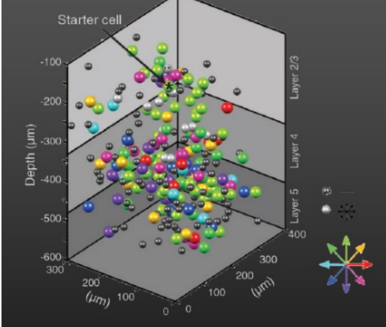


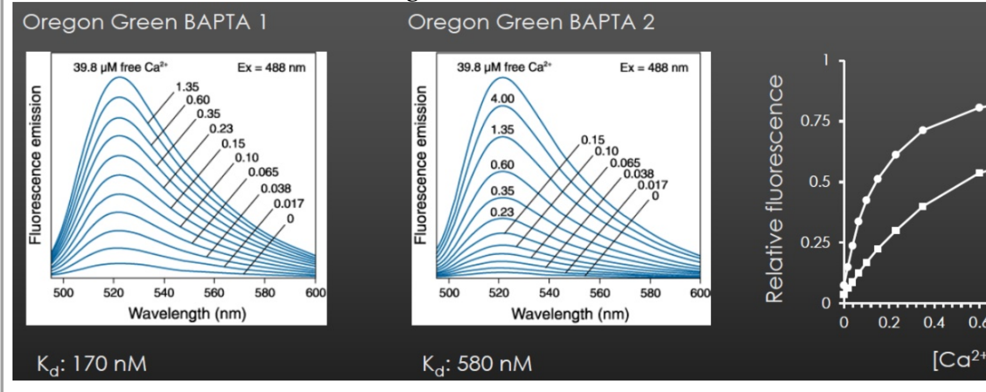
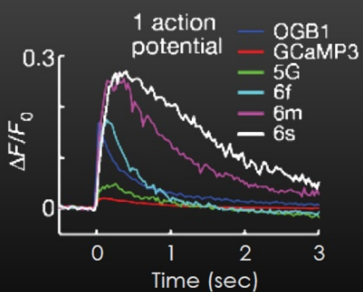
- Virus is able to travel retrogradely and travel through synapses
- You can make a virus that can only travel retrogradely once -> avoid marking all the network (protein G is present in the plasmid and only available in the soma of the starter cell) -> Imaging of presynaptic neurons
- You can make the virus express the gene of interest

With this technique, you can visualize all presynaptic neurons from a single starter neuron



Example data

- Postsynaptic neurons has a prefential motion activation pattern, but it also receives inputs from neuron with other motion patterns (challenge - overcome those inputs)
- 

<table>
<tr><td>Which techniques can you use to label whole networks?</td><td>Labeling entire networks
Bolus loading
Transgenic mice
Viral injections</td></tr>
<tr><td>Which one is better: OGB1 or OGB2? Why?</td><td>Choice of indicators and quantification
Oregon Green BAPTA 1 - Kd: 170 nM (high affinity, senses calcium at low concentrations, saturates quickly at lower concentrations of Ca2+)
Resting Ca2+ concentration in the cytosol of neurons - 50-100 nM
Oregon Green BAPTA 2 - Lower affinity, takes more calcium before it saturates
Lower impact on endogenous buffer Ca2+
Linear representation of amplitude
Better kinetics at higher concentrations

</td></tr>
<tr><td>What the currently available types of genetically encoded calcium indicators?</td><td>Genetically encoded calcium indicators
GCaMP3 - High coeffient (the signal is non-linear)
GCaMP6 - Slow (best signal to noise, slow temporal resolution); fast (worst signal to noise, better temporal resolution)


Latest generation
jGCaMP7 - More linear signal (Dana et al 2019)</td></tr>
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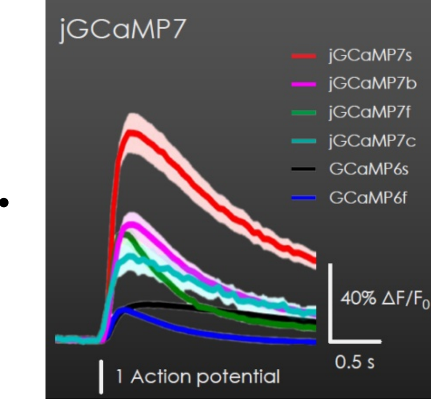
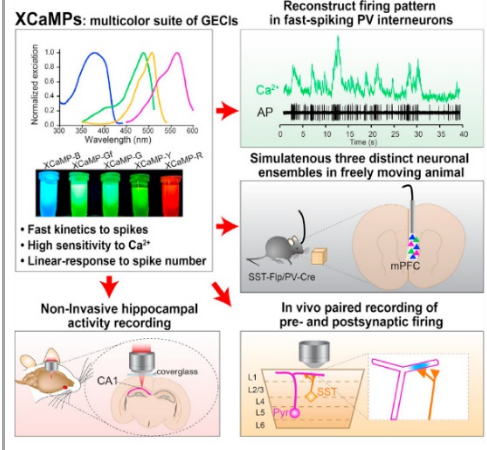
- 

- XCaMPs - Multicolor suite of GECI, even more linear (Inoue et al 2019)



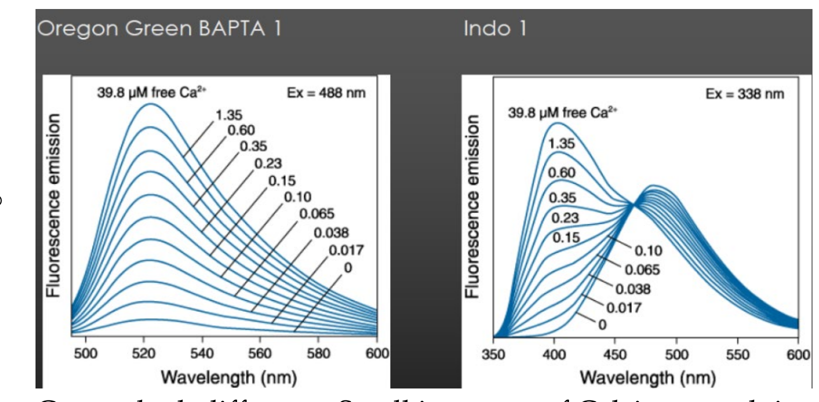
</td></tr>
<tr><td>How can one correct for drifting effects in GCaMP data?</td><td>

Preparation of the data for analysis:

- Drift correction - Problem with region of interest calculation if the neuron is moving around the frame
  - XY drift is correct, there is still some Z drift (the neuron is moving up and down); can be corrected by z-stacking
- **Simons Foundation tools for calcium imaging**
</td></tr>
<tr><td>How can you extract data from miniscope videos?</td><td>

Data from images

- Defining ROI
- Calculating DeltaF/F based on baseline
  - It is difficult to compare DeltaF/F between different animals and different experiments
- If calibrated correctly, you can estimate the calcium concentration
</td></tr>
</table>



- Curves look different - Small increases of Calcium result in large increases of fluorescence

What are the advantages of ratiometric indicators compared to intensiometric ones?

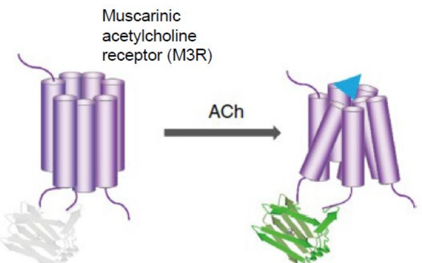
Advantages of ratiometric indicators

- Even when the neuron jumps out of focus, the ratio of peaks stays the same -> Artifacts are limited
- Required two wavelengths
- Can be used in two photon microscopy on the emission side, not the absorption

	Single-wavelength	Ratiometric
Examples	Oregon Green BAPTA 1 GCaMP6	Fura-2, Indo 1
Analysis	$\Delta F/F_0$	Wavelength 1/ wavelength 2
Determination of $[Ca^{2+}]_i$	Difficult	Simple calibration
Recording	1 Wavelength	2 Wavelengths, slow
Artifacts (movements, bleaching	Present	Little
Choices	Large selection	Only UV excitation for synthetic dyes

Novel sensors for transmitters and modulators

- Acetylcholine sensor - Same principle, but fluorescence increases with Ach

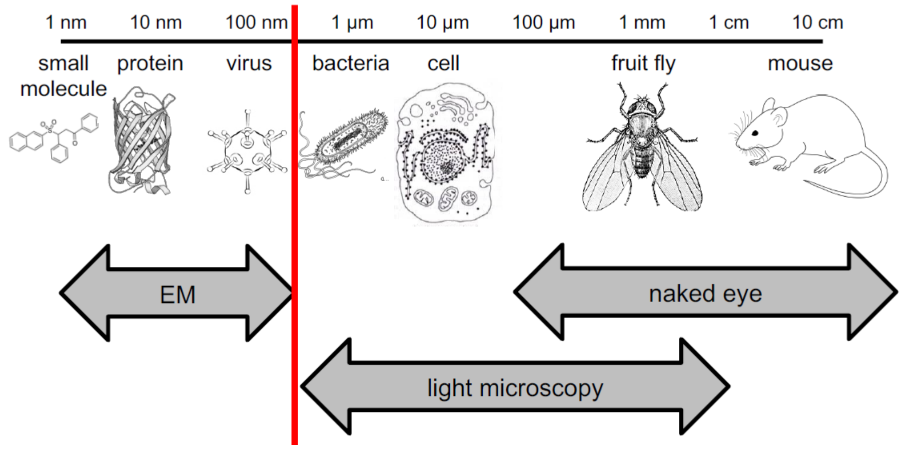
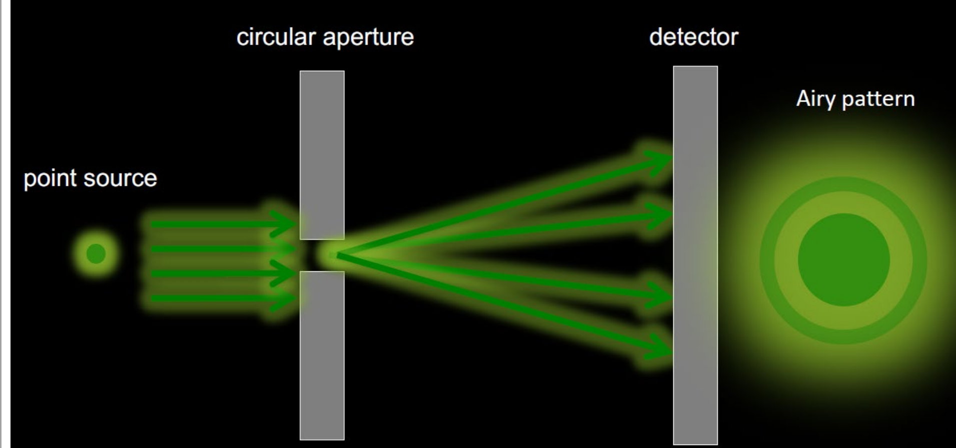


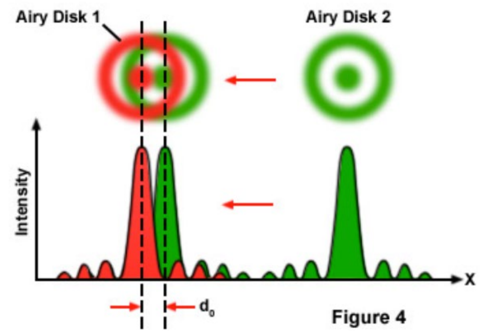
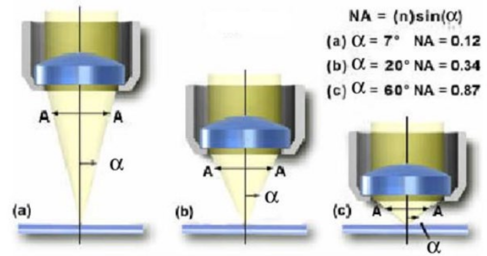
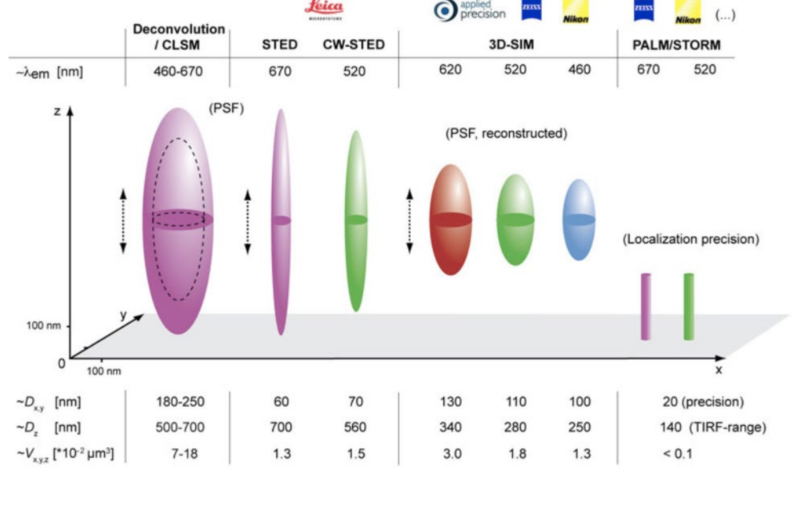
- Signal can also be blocked with Ach antagonists

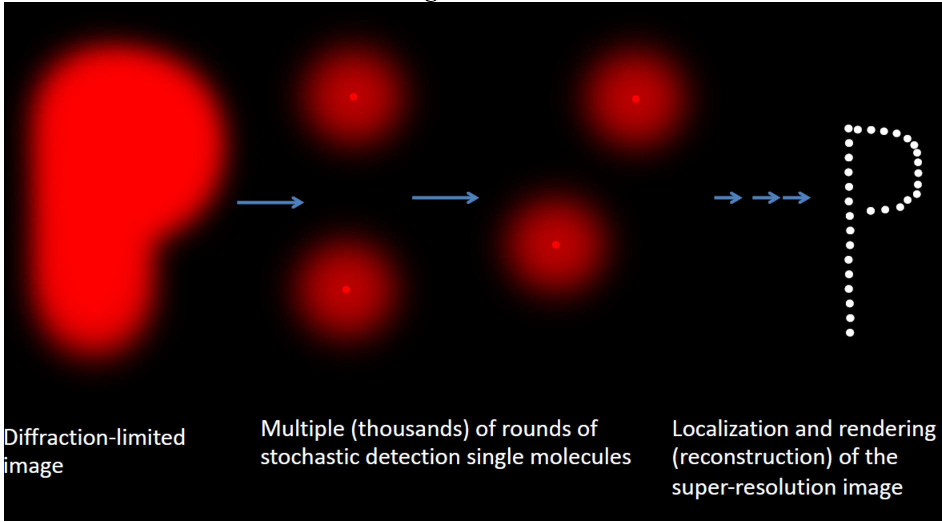
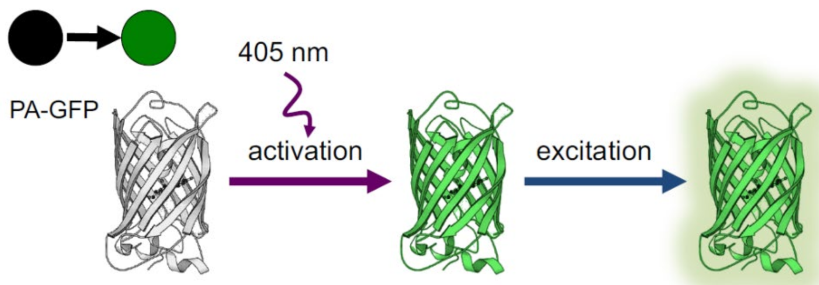
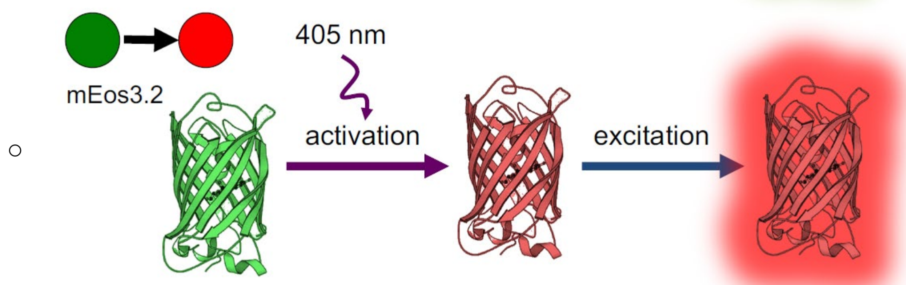
Software for image analysis

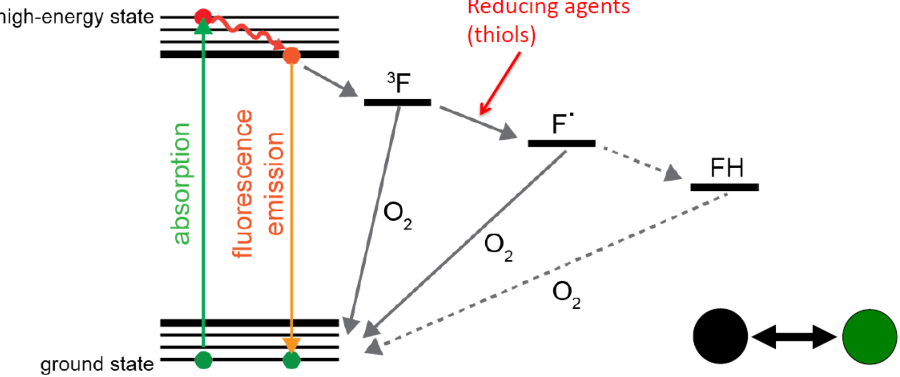
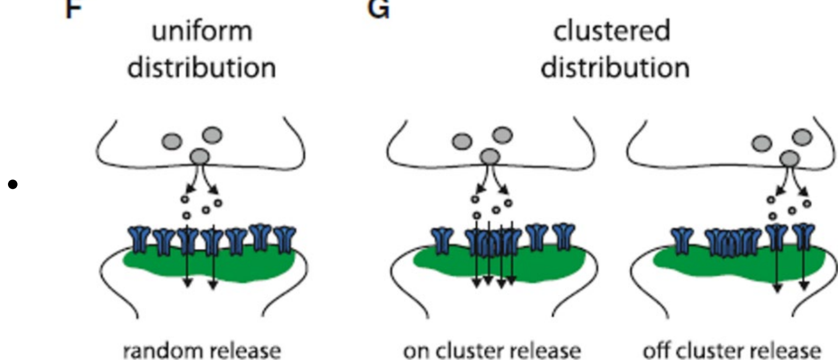
- ImageJ
- MATLAB vs Python

Super-resolution microscopy - Mapping and tracking protein distributions in neurons (Harold D. MacGillavry)

What is the main advantage of light microscopy over electron microscopy?	Molecular anatomy of excitatory synapses • Alignment of the presynapse with the postsynapse - release site needs to be close to receptors in the postsynapse • Live cell imaging - allows to see synaptic changes in real-time (as opposed to EM and fixed tissue) 
What is the main limitation of light microscopy?	Diffraction limit - half of the wavelength of light • Does not allow for the resolution of organelles/structures - They combine to a single pixel of resolution • Where the problem comes from: diffraction of light - single points become airy discs; Abbe's Law - $d = \lambda / 2*n*sin(a)$  ○ Longer wavelength - More diffraction, less resolution ○ The final resolution of image depends on much more: alignment of the optical system, structure/type of specimen, optical aberrations, stage drift

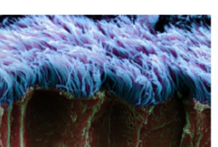
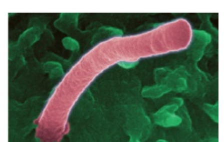
What is resolution?	Resolution - Shortest distance between two points that can still be distinguished 
How is possible to break the diffraction barrier?	Breaking the diffraction barrier?  • Increase NA - Oil barrier objective • 4pi microscopy - two objectives to collect more light • Improve analysis • Improve illumination - STED (30-70 nm, high LASER power), SIM (150 nm) • Single-molecule localization microscopy - PALM (10-30 nm), STORM, PAINT (all of them are currently known as single molecule tracking techniques)
What are the resolving power of super-resolution techniques?	Resolving power of super-resolution techniques 

What is the principle behind single molecule imaging (PALM and STORM)?	Single molecule imaging • Define points from the point spread function observed • Localization precision - Proportion to the number of photons, inversely proportional to the standard deviation of the PSF • Overall principal - Instead of looking at all light points at once, you separate the emission over time; then stack the frames together to reconstruct the overall image 
What is the principle of PALM?	PALM - Photoactivatable light microscopy  • Uses a variety of GFP that only shines when UV light is emitted to the sample • Low power light source - Only activates a few fluorophores at a time • **Photoconvertable fluorophores - Emit different wavelengths when activated** ◦  • mEOS - It only activates once (the same fluorophore is not activated twice)

What is the principle behing STORM?	STORM - Stochastic switching of organic fluorescent dyes  • Triplet states - Dark states, molecules can enter these states spontaneously; Can be stimulated by using reducing agents; It spontaneously recovers via light-induced oxidation • Similarly to PALM, results in stochastically blinking • Quality of sample preparation is really important - You might observe gaps in the final image, when the structure is actually continuous ○ Since this works with antibody labeling, it is possible that some portions are not possible to be labeled (oclusion from the antibody) • STORM provides 100x more photons than PALM -> has higher resolution
What is the main application for PALM?	Examples of study • Single molecule localization microscopy reveals heterogenous distribution of synaptic proteins •  • Single molecule tracking - Rapid and consecutive PALM snapshots ○ Receptors are confined to certain regions of the perisynaptic sites

Active transport in *C. elegans* sensory neurons (Jaap van Krugten)

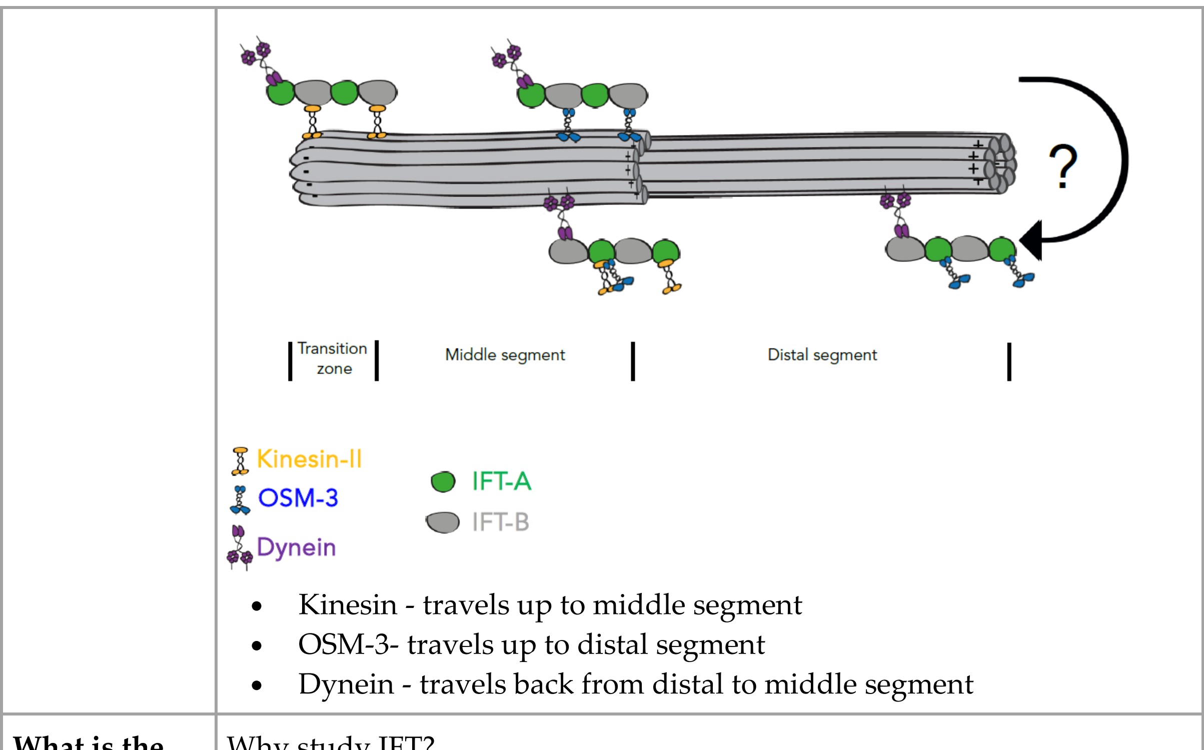
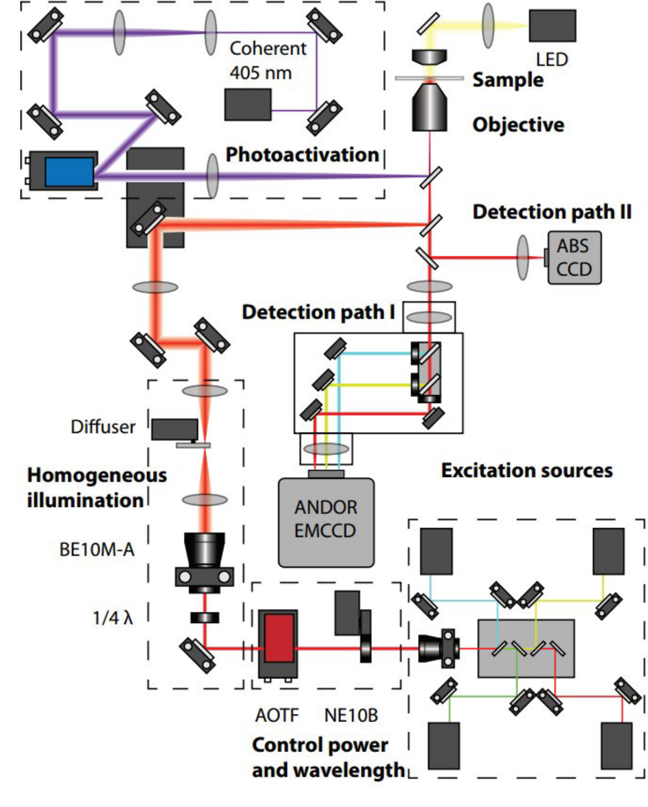
<table>
<tr>
<td>Why is C. elegans a good model organism for neuroscience?</td>
<td>C. Elegans as a model organism

1 mm long, 952 cells; 302 neurons
Transparent body
Known genome, cell cycle and connectome
Fast life cycle - Adult worms in four days
Easy maintenance, able to freeze for years
</td>
</tr>
<tr>
<td>What are cillia? How many C. elegans neurons are ciliated?</td>
<td>Cilium - hair-like structure or organelle extending from the surface
Motile cilium Non-motile cilium



Motile cilium - Motor function
Non-motile (primary) cilium - Sensory function
From the 302 neurons, 60 are cilliated
</td>
</tr>
<tr>
<td>What are the roles of cilia in C. elegans?</td>
<td>Cilia are found at the tips of sensory neurons

The end of cilia are opened to the outside
Roles: chemoattraction and repulsion, thermosensation, mechanosensation, oxygen sensing
</td>
</tr>
<tr>
<td>Describe the roles of kinesin-II, OSM-3 and dynein in building the cilium.</td>
<td>Cilium is built and maintained by IFT (intraflagellar transport)

Middle segment - Microtubule doublet (A and B)
Distal segment - Microtubule singlet (A)
</td>
</tr>
</table>

	 • Kinesin - travels up to middle segment • OSM-3- travels up to distal segment • Dynein - travels back from distal to middle segment
What is the utility of studying intraflagellar transport?	Why study IFT? • It is interesting • Some human diseases are related to IFT mutation (ciliopathies) -> most are related to the transition zone
Describe the general setup of the widefield microscope used to visualize *C. elegans*.	**Microscope setup** 

	EMCCD (electron multiplier) - Good to reduce dark noise CMOS tend to be less sensitive, though higher in framerate Laser alignment is very difficult -> you need two mirrors to precisely direct the beam Beam expander - Necessary to increase the diameter of the beam 1/4 waveplate - Serves to polarize the light, increase range of detection Optosplit - Dichroic mirrors that separate light before they reach the detector
What are some practical consideration in the use of *C. elegans* - as opposed to fixed tissue?	Practical considerations of fluorescent imaging of living sample • Endogenous expression (MoSCI/CRISPR-Cas9) -> one to one expresssion; more difficult, take longer than overexpression • Overexpression (injection/transfection) -> lots of copies of fluorescent proteins; not adaquete for transport studies (excess proteins disrupt the dynamics) • Tissue is not fixed - Anaesthesia is important to keep the worms still (placed on top of a agarose pad) Anaesthetic Anaesthesized worms on agar pad
Why is TIRF not a feasible technique for *C. elegans*?	C. Elegans is a round, not flatworm • TIRF is not possible (evanescent wave is only two hundred nanometers thick)
What can be learned when using a *C. elegans* as a model organism?	What can we learn with this technique? • Track and quantify movement of proteins in living organisms Example research (not on exam) • IFT-Dyenin tagged with GFP - Dyenin goes out of the distal segment once the repelent is added in the medium • OCR-2 tagged with GFP - Unchanged once repelent is added Violin plot