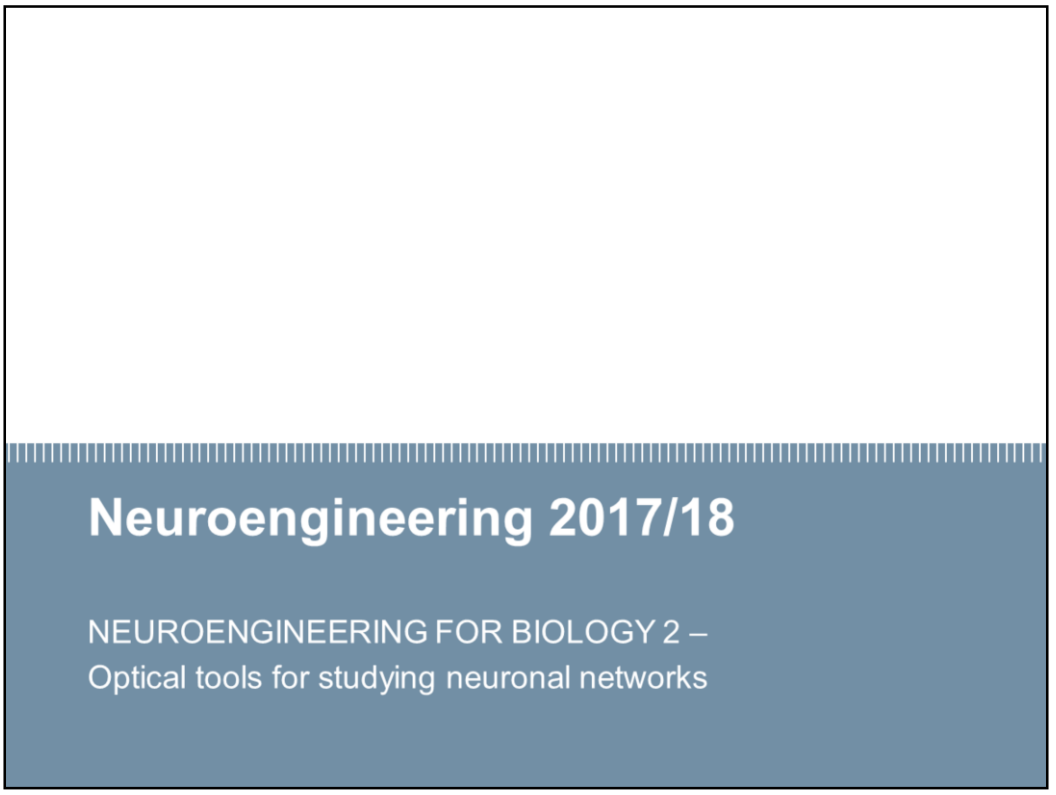


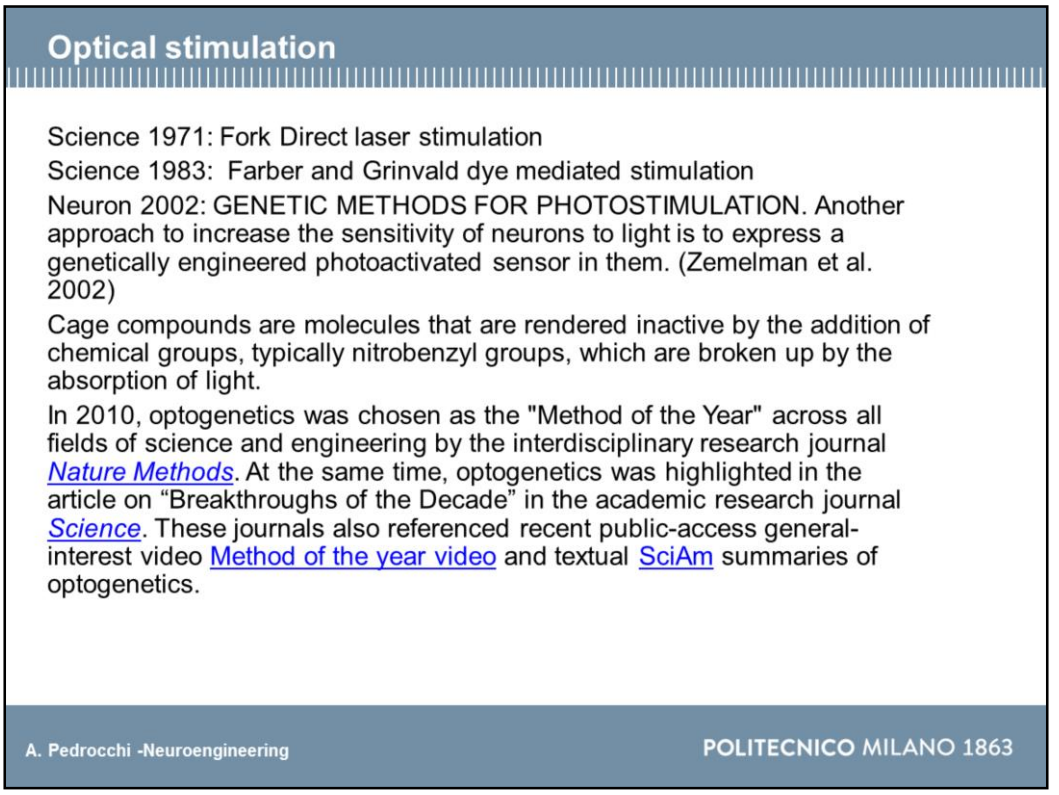
OPTICS:

major model to interact

optical stimulation, they started to understanad that you can stimulate neurons by lighting them, and the main advantage is that light is **very NON INVASIVE** and **very easy to move** because of fiber optics.. then there have been 2 directions for optical stimulation:
1) in vitro:
cage compounds. they are added to the medium of a culture, and they can block some of the molecules that are inside the culture.
*Praticamente: tu inserisci queste cage compounds, e loro si mischiano all'interno del medium e vanno un po' ovunque.

But if you have cage compouns which can be released by light you can very accurately activate them by using optical stimlation. So we have like glutammate.. typical excitatory neuraltrasmitter in the brain. I put glutammate in the culture everyone is activated. If I can cage the gluttamete I can put it in the medium and nothing happens because it doesnt excape. then with a light, very accurately focused on one area of my culture, I can uncage the GLUTAMMATE only in that area. So If I find a cagecompound wich can be cut by light i can make a very selective optical stimulation. So cage compounds are based on system which once bounded they make the molecules inactive. If you can cut them the molecules does what's its original goal.

The point is: there are many different cage compounds that can be cut by light!THIS IS CALLED OPTICAL STIMULATION. BY OPTICS YOU ACTIVATE IN A VERY SELECTIVE AREA, THE DIMENSION DEPENDS ON THE FIBER OPTICS, BUT YOU CAN ACTIVATE THE COMPOUND. And this is the principle of optogenetics. It uses molecules based on a change on the DNA. Not on humans btw for now.



Primer on Optogenetics: Pastrana, Erika (2010). "Optogenetics: Controlling cell function with light". Nature Methods 8 (1): 24. doi:10.1038/nmeth.f.323.
Editorial: "Method of the Year 2010". Nature Methods 8 (1): 1. 2010. doi:10.1038/nmeth.f.321.
Commentary: Deisseroth, Karl (2010). "Optogenetics". Nature Methods 8 (1): 26–9. doi:10.1038/nmeth.f.324. PMID 21191368. News, Staff (2010). "Insights of the decade. Stepping away from the trees for a look at the forest. Introduction". Science 330 (6011): 1612–3. Bibcode:2010Sci...330.1612.. doi:10.1126/science.330.6011.1612. PMID 21163985.

Video link http://www.youtube.com/watch?v=I64X7vHSHOE

Another interesting video: https://youtu.be/Nb07TLkJ3Ww

So far optogenetics has had a lot of impact in the scientific world. But it hasn't been used in any human patients yet. There are a couple reasons why. One is that it requires a gene therapy to deliver the gene that encodes for these light activated molecules into the body. Currently in the U.S. there are no FDA approved gene therapies. In Europe there's just one. Another issue is that these molecules come from organisms like algae and bacteria. And so if we are putting these molecules into the body would they be detected as foreign agents and attacked by the immune system, for example.

Published on Nov 7, 2013

Comment: the reason not to go deeper in the study of optogenetics but to at least mention it in this class, is because it is having a great impact nowadays in research in neuroscience so it's worthy to know its principle. But at the moment, the most critical methodological issue about this technique are in the field of biotechnology and genetic engineering, which are biologists' tasks (non engineers', despite the terms).

Neuroengineering 2017/18

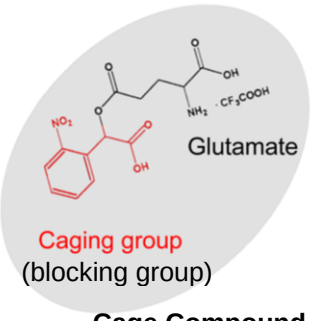
NEUROENGINEERING FOR BIOLOGY 2 –
Optical tools for studying neuronal networks
PART 1–
Optical stimulation of in vitro neuronal cultures

Optical stimulation

ONE-PHOTON UNCAGING

The basic approach is to cage the compound (Black) with a blocking group (red). Thus, caged compound can be switched into the active form by short UV pulses. **short pulses:50ms, 10ms**

This allows us to obtain high spatial and temporal control during stimulation.



Cage Compound

PROS

- Glutammate is one of the most common neurtransmitters in the CNS

  → Physiological stimulation

- The UV pulse can be highly focused

  →High selectivity

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Optical stimulation uses photolabile neurotransmitters. For instance, chemists can modify the glutamate molecule, placing a compound that inactivates it. If the bond between the Glutamate and the inactivating compound is photolabile, then it is possible to separate the two molecules with light stimulation. Once the Glutamate is uncaged (i.e. the bond is broken), the stimulus to the neurons is the same as the natural one.

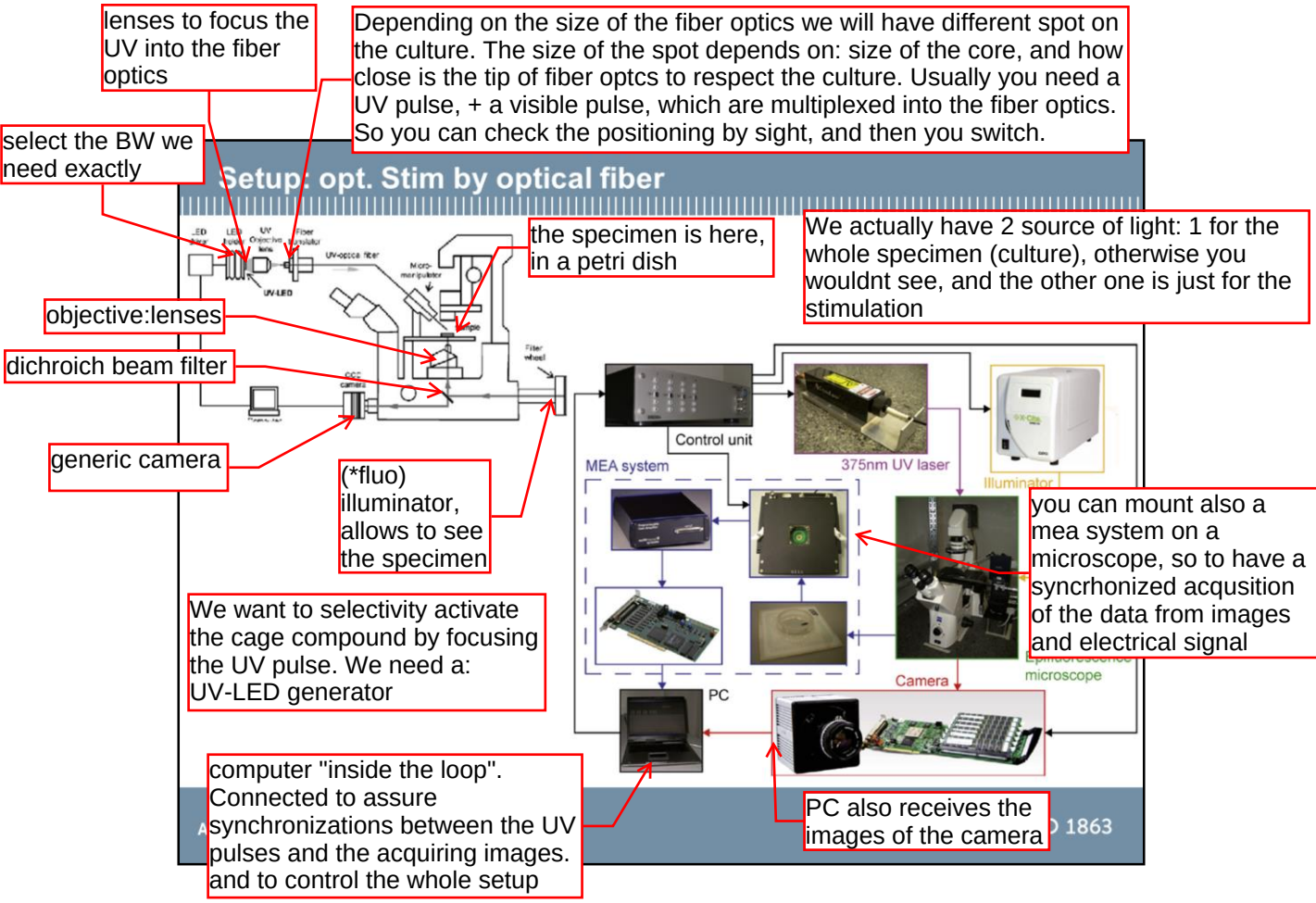
The question now is: how selective is the optical stimulation?

Use of photolabile compounds.
photolobial neurotrasmitter: compound which has a blocking group, the compound is the N.Transmitter, and is caged, so blocked, by a blocking group.

The cage compound can be switched, into the active form, by using light. Usually UV pulses. That's why " photo labile", because by light you cut "the cage part" the blocking group, and you have the origianl compound, which is a neural transmitter and thus it's active.

[6.30]
Advantage of cage compound:
1st: stimulation absolutely physiological, it's based on neural transmitter. It's not like an electrical stimulation, which is talking to neurons which are used to "talk" to each others by chemicals (neuro trans.)! While indeed here you use the neurotrasmitter that are present in a standard physiological situation.

2nd: you can achieve a very high selectivity.
How can you be selective in focusing the UV pulses? the basic system that you need is : following slide



Essential set up for optical stimulation

It is possible to use an optical fiber that focuses the stimulus on the culture.

The uncaging optical stimulus, generated by the UV LED, is carried through a fiber optics which is placed on the sample by mean of micromanipulator.

The image of the sample is illuminated by the Xenon Arc lamp. The sample image is conveyed by the optics of a direct microscope. The objective is underneath the slide. The image is recorded by the CCD camera which is syncronized with the LED through the controlling system (PC).

Fig. 2. Optical stimulation combined with MEA recording

General scheme of the developed set-up. The UV laser, the fluorescence illuminator, the camera and the MEA systemare temporally timed by a software programmable

TTL control unit (black lines). A 375nm laser is used to uncage the compound on the sample through the micromoved optical fibre (pink lines), whereas the responses induced by optical uncaging are detected either via electrical recordings by a MEA system (blue lines) or via inverted epifluorescence microscope fluorescence measurements by a CMOS intensified camera (red lines). Laser output was focused into a single mode UV optical fibre ~~(2m core and 125m clad with 0.12NA, BC1197,~~

~~OZ Optics, Ottawa, Canada) resulting in a power output of 1mW.~~ The optical fibre was mounted on a computer assisted micromanipulator ~~(Physik Instrumente, Karlsruhe, Germany),~~ thus allowing placing and moving easily the fibre tip in the areas of interest of the culture.

~~REFERENCE Ghezzi et al., A Micro-Electrode Array device coupled to a laser-based system for the local stimulation of neurons by optical release of glutamate Journal of Neuroscience Methods 175 (2008) 70–78~~

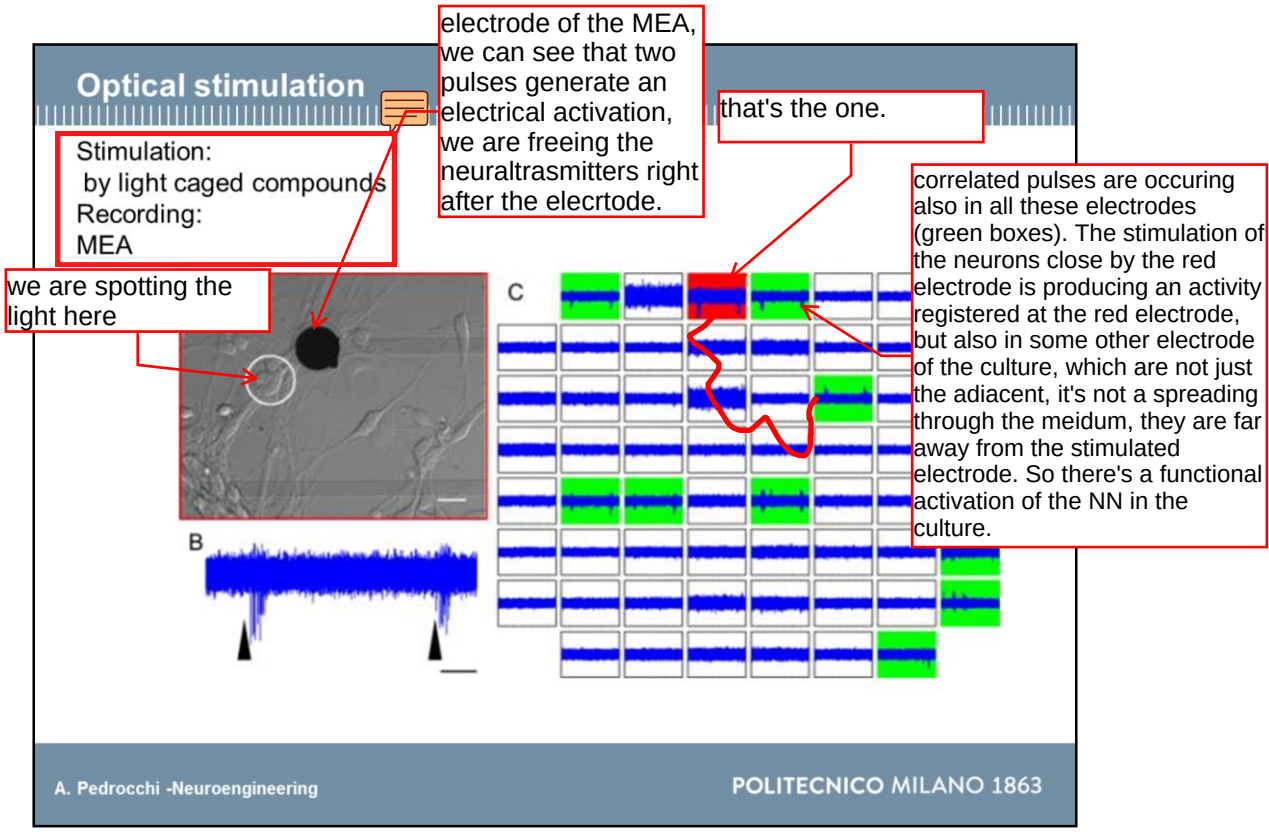
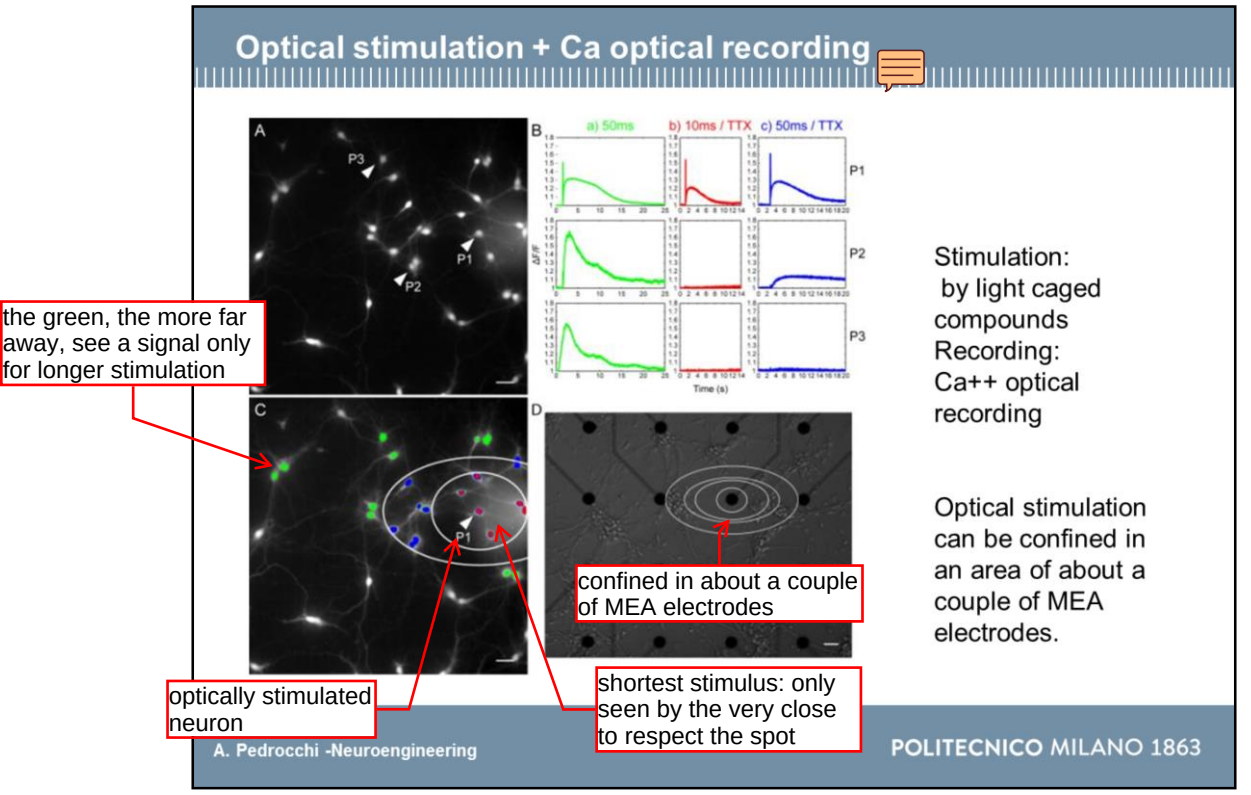


Figure 9: Local stimulation of a site of the neuronal network and detection of the resulting activity on the whole network. (*Panel A*) The optically stimulated area near one of the sixty electrodes is marked by a white circle. (*Panel B*) Activity recorded in the nearest electrode after two optical pulses of 50ms. The application of the pulses is indicated by the black arrow heads. (*Panel C*) Activity recorded from the entire network. The red box highlight the trace of the electrode very close to the stimulus area. The green squares represent the areas where there is correlated activity. We can observe that the synchronous activity doesn't reflect the passive spatial morphology [e.g. electrical field decreasing with distance]. This demonstrates that the culture has a network activity that can be recorded: we have a functional recording.

Note – the figure in the slide represents the topology of the electrodes with registrations in a given temporal window.



It is necessary to determine the area of the culture stimulated by the optical fiber. Through optical methods, it is possible to determine it exactly [see slide].

Figure 7: Optical recording of $[Ca^{2+}]_i$ variations following glutamate uncaging elicited by UV light pulses of various durations ~~in presence of MNI-caged-L-glutamate (100µM). (*Panel A*)~~ The fluorescence picture shows the field of view of the stimulated network where neurons are labelled with Fluo4-AM. The optically stimulated neuron is indicated by the P1 marker. (*Panel B*) $[Ca^{2+}]_i$ variations of three selected neurons (P1, P2 and P3) under three different conditions a) Green: pulse of 50ms; b) Red: pulse of 10ms in presence of TTX 2µM; c) Blue: pulse of 50ms in presence of TTX 2µM – note: Tetrodotoxin (TTX) is a potent neurotoxin, if added to the medium of culture it reduces the overall activity of the neurons.). (*Panel C*) The graphical representation of the neurons responding in the three different conditions shows the dimension of the excited area. The red markers represent neurons (i.e. P1) responding in all the three conditions. The blue markers represent neurons (i.e. P2) responding both in the a) and c) conditions and the green markers represent neurons (i.e. P3) responding only in the a) condition. The white circles identify the dimension of the stimulated area for pulses respectively of 10ms and 50ms measured in presence of TTX 2µM. (*Panel D*) The white circles indicate the stimulated area thus obtained for different pulses of 5ms, 10ms, 30ms and 50ms. This extension is obtained at a mean of n=5 trials for every pulse duration. The superimposing of the white circles with a neuronal culture on a MEA shows the practical extension of the stimulation for different pulse durations. ~~The scale bar is 40µm.~~

Neuroengineering 2017/18

NEUROENGINEERING FOR BIOLOGY 2 –
Optical tools for studying neuronal networks
PART 2–
Optical recording of in vitro neuronal cultures activity

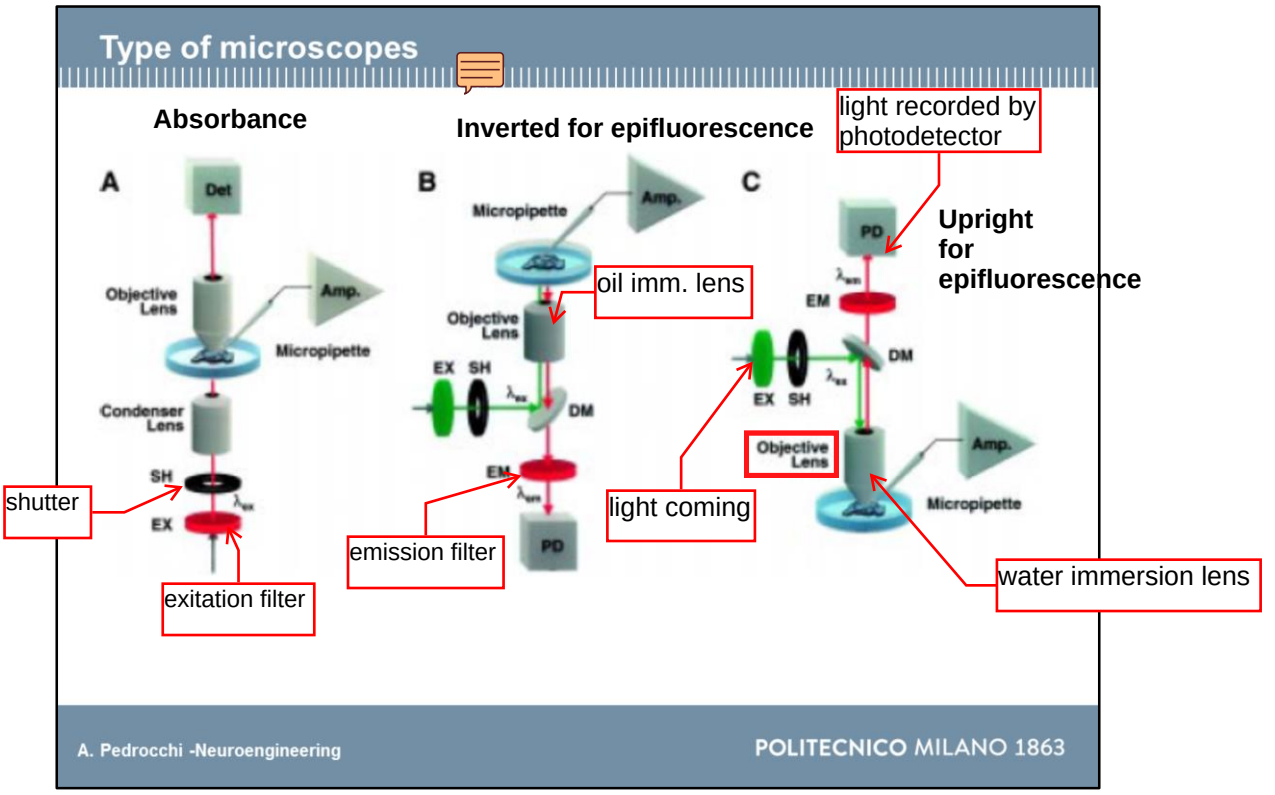


Fig. 1. Schematics of typical optical recording setup. **A.** Microscope for absorbance measurement. Light is limited by an excitation

filter (EX) to the appropriate wavelength (li), an electromechanical shutter (SH) to control the time of illumination and

is directed onto the preparation via a condenser lens. Transmitted light is collected by the objective lens (water-immersion)

and quantified by a photodetector (Det). **B.** Inverted microscope for epifluorescence measurement. Excitation light (lex) is directed

onto the preparation by a dichroic beamsplitter (DB) and the objective lens (air or oil-immersion). Fluorescence emitted

from the preparation (lem) is collected by the same lens, passes the DB, is filtered by the emission filter (EM) and quantified

by DET. **C.** Upright microscope for epifluorescence measurement. Setup is analogous to that in B, except that it is now in an

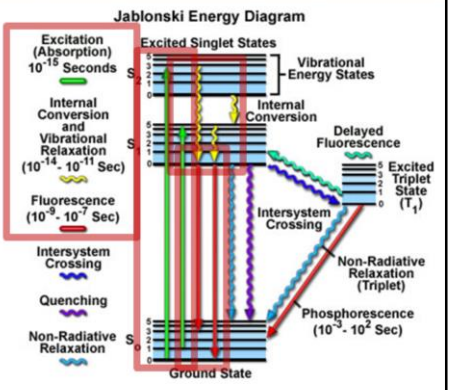
upright configuration, utilizing a water immersion lens.

Optical recording need microscopes, and usually we have 3 major cathegory:
1) for absorbance
you have the light, which goes thourgh the lesens, passes in the specimen, then the image are aquiried by the objective wich is in the other side, then the camera.
2) inverted microscope (b)
both the light goes to the specimen, then the reading pass thorugh the same direction, going back to the camera.
3) upright microscope with epifluorescence
similar to the inverted but goes to the upright.

Fluorescence

Fluorescence is governed by three events:

- Excitation (or absorption)
- Vibrational relaxation
- Emission



Fluorescence is a quick process: it is measured in billionths of seconds

Energy loss in vibrational relaxation causes emitted photon to have less energy than absorbed one

Emitted photon has a different wavelength ("color") than absorbed one

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main feature read: based on fluorescence.

-- Short refresh about fluorescence:
[references in the notes]
process which allows a specimen to absorb light, to change the status of energy, as a consequence of the light which is exciting the compound, and then going back to the initial state by emitting a ligh which is ALWAYS at a lower energy, meaning longer wavelenghts.

So we have: excitation, vibrational relaxation and then emission. During the change of state we have a loss of energy. So exc. is always higher than emission energy.
If we have some compounds which are fluorescent at different wavelenghts, they can be selectively excited. The trick is based on biotechnology: they can develope compounds which binds to specific structures, so you can illuminate at the proper excitation wl, and have an image on only the structure which is bounded. You want to see Ca++ going inside and outside a synapses, you must find a flur. compound which binds a Ca++. then you excite wht the w.l of that compound.

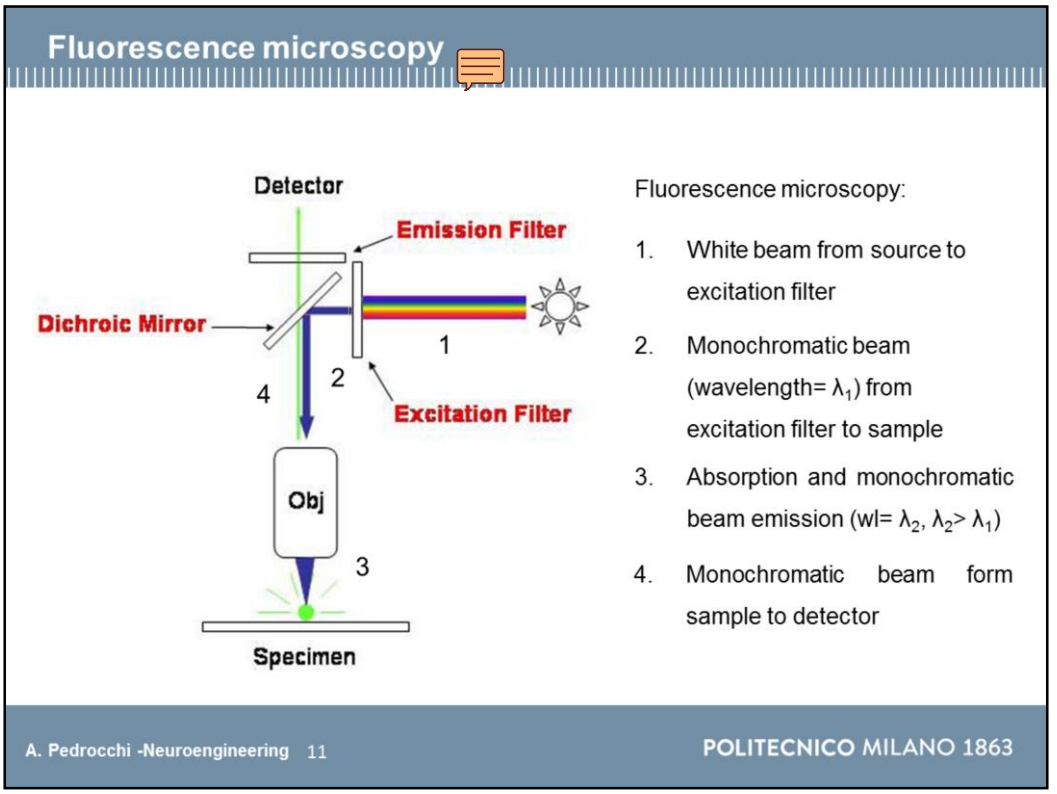
COMPOUNDS THAT BINDS SELECTIVITY TO THE STRUCTURE THAT YOU WANT TO STUDY. each compound does have a specific excitation and emission wavelength.

Playing with the emission and excitation you can activate one or the other. You can put multiple compund in the medium. So then use one or another ligth to see one or another compond.

A variety of specimens exhibit autofluorescence (without the application of fluorochromes) when they are irradiated, a phenomenon that has been thoroughly exploited in the fields of botany, petrology, and the semiconductor industry. In contrast, the study of animal tissues and pathogens is often complicated with either extremely faint or bright, nonspecific autofluorescence. Of far greater value for the latter studies are added fluorochromes (also termed **fluorophores**), which are excited by specific wavelengths of irradiating light and emit light of defined and useful intensity. **Fluorochromes are stains that attach themselves to visible or sub-visible structures, are often highly specific in their attachment targeting, and have a significant quantum yield (the ratio of photon absorption to emission).** The widespread growth in the utilization of fluorescence microscopy is closely linked to the development of new synthetic and naturally occurring fluorophores with known intensity profiles of excitation and emission, along with well-understood biological targets.

Stokes' Shift

Vibrational energy is lost when electrons relax from the excited state back to the ground state. As a result of the energy loss, the emission spectrum of an excited fluorophore is usually shifted to longer wavelengths when compared to the absorption or excitation spectrum (note that wavelength varies inversely to radiation energy). This well-documented phenomenon is known as **Stokes' Law** or **Stokes' shift**. As Stokes' shift values increase, it becomes easier to separate excitation from emission light through the use of fluorescence filter combinations.



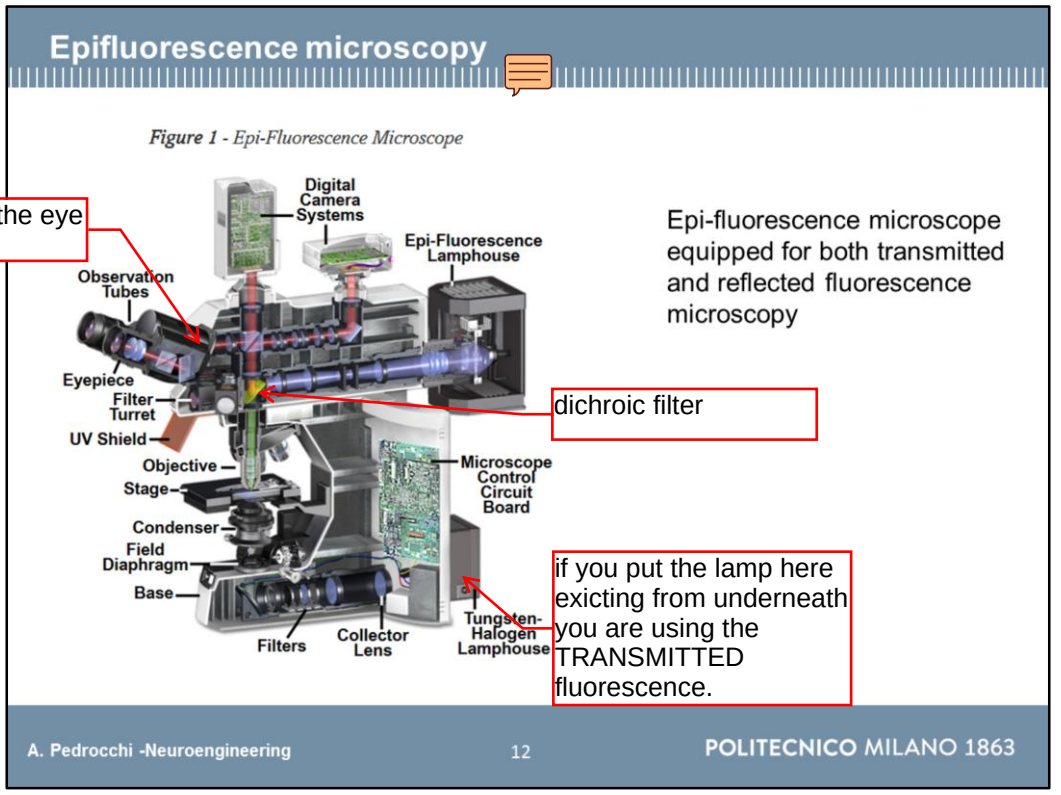
FOR MORE DETAILS (beyond exam content)
https://www.microscopyu.com/techniques/fluorescence/introduction-to-fluorescence-microscopy)

Basis:
1. excitation source(light source), the sun in the image. A white lamp we have all the wl.s. So we need an excitation filter, which select exactly the WL. that we need, to select the compound we want. (2)

Then dichroic filter: special filter which reflects shorter wl and let pass longer wl. Ofc you choose excitation filter and dichroic mirror depending on the exact wl you want, so depending on the fluorescent compound you are using.

So the excitation filter goes to the objective, and then it's focused on the compound. Then the flourescence molecule is excited and start emitting at the longer wavelength. The emission is not focused. It goes in every direction. So the objective can capture emission, but this emission DOES pass thorugh a dichroic mirror (obv. properly chosen, allows emission to pass thorugh, excitation to be reflected).
Emitted light so goes in the detector.
Before the detector there's usually an emission filter to be sure, because the dichroic mirror just let pass lower wl. than something.. so it's better to filter again.

Emitted light is a TINY signal to respect the excitation.



it goes also to the eye tools

dichroic filter

if you put the lamp here exicting from underneath you are using the TRANSMITTED fluorescence.

Fundamentals of Excitation and Emission

The basic function of a fluorescence microscope is to irradiate the specimen with a desired and specific band of wavelengths, and then to separate the much weaker emitted fluorescence from the excitation light. In a properly configured microscope, only the emission light should reach the eye or detector so that the resulting fluorescent structures are superimposed with high contrast against a very dark (or black) background. The limits of detection are generally governed by the darkness of the background, and the excitation light is typically several hundred thousand to a million times brighter than the emitted fluorescence.

Illustrated in **Figure 1** is a cutaway diagram of a modern epi-fluorescence microscope equipped for both transmitted and reflected fluorescence microscopy.

The vertical illuminator in the center of the diagram has the light source positioned at one end (labeled the episcopic lamphouse) and the filter cube turret at the other. The design consists of a basic reflected light microscope in which the wavelength of the reflected light is longer than that of the excitation. Johan S. Ploem is credited with the development of the vertical illuminator for reflected light fluorescence microscopy. In a fluorescence vertical illuminator, light of a specific wavelength (or defined band of wavelengths), often in the ultraviolet, blue or green regions of the visible spectrum, is produced by passing multispectral light from an arc-discharge lamp or other source through a wavelength selective **excitation** filter. Wavelengths passed by the excitation filter reflect from the surface of a **dichromatic** (also termed a **dichroic**) mirror or beamsplitter, through the microscope objective to bath the specimen with intense light. If the specimen fluoresces, the emission light gathered by the objective passes back through the dichromatic mirror and is subsequently filtered by a **barrier** (or **emission**) filter, which blocks the unwanted excitation wavelengths. It is important to note that fluorescence is the only mode in optical microscopy where the specimen, subsequent to excitation, produces its own light. **The emitted light re-radiates spherically in all directions, regardless of the excitation light source direction**.

Epi-fluorescence illumination is the overwhelming choice of techniques in modern microscopy, and the reflected light vertical illuminator is interposed between the observation viewing tubes and the nosepiece housing the objectives. The illuminator is designed to direct light onto the specimen by first passing the excitation light through the microscope objective (which in this configuration, acts as a **condenser**) on the way toward the specimen, and then using that same objective to capture the emitted fluorescence. This type of illuminator has several advantages. The fluorescence microscope objective serves first as a well-corrected condenser and secondly as the image-forming light gatherer. Being a single component, the objective/condenser is always in perfect alignment. A majority of the excitation light reaching the specimen passes through without interaction and travels away from the objective, and the illuminated area is restricted to that which is observed through the eyepieces (in most cases).

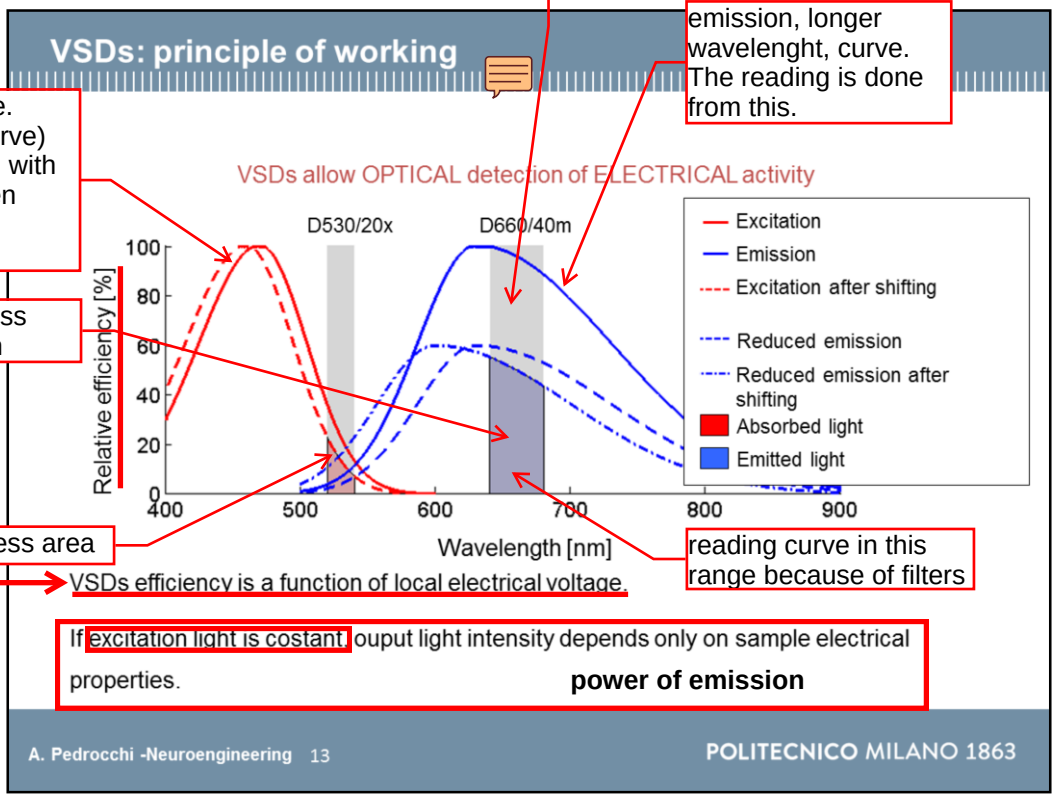
Finally, it is possible to combine with or alternate between reflected light fluorescence and transmitted light observation and the capture of digital images.

As presented in **Figure** , the reflected light vertical illuminator comprises an arc-discharge lamphouse at the rear end (usually a mercury or xenon burner). Excitation light travels along the illuminator perpendicular to the optical axis of the microscope, passes through collector lenses and a variable, centerable aperture diaphragm, and then through a variable, centerable field diaphragm. The light then impinges upon the excitation filter where selection of the desired band and blockage of unwanted wavelength occurs. The selected wavelengths, after passing through the excitation filter, reach the dichromatic beamsplitting mirror, which is a specialized interference filter that efficiently reflects shorter wavelength light and efficiently passes longer wavelength light. The dichromatic beamsplitter is tilted at a 45-degree angle with respect to the incoming excitation light and reflects this illumination at a 90-degree angle directly through the objective optical system and onto the specimen. Fluorescence emission produced by the illuminated specimen is gathered by the objective, now serving in its usual image-forming function. Because the emitted light consists of longer wavelengths than the excitation illumination, it is able to pass through the dichromatic mirror and upward to the observation tubes or electronic detector.

Most of the scattered excitation light reaching the dichromatic mirror is reflected back toward the light source, although a minute quantity often passes through and is absorbed by the internal coating of the mirror block. Before the emitted fluorescence can reach the eyepiece or detector, it must first pass through the barrier or suppression filter.

Modern fluorescence microscopes are capable of accommodating between four and six fluorescence cubes (usually on a revolving turret or through a slider mechanism; and permit the user to easily attach replacement aftermarket excitation and barrier filters, as well as dichromatic mirrors.

VOLTAGE SENSITIVE DYES



area during spike off: power emission is high

emission, longer wavelenght, curve. The reading is done from this.

Excitation curve. (*Absorption curve) We are exciting with less power when there's spiking (shifted curve)

spiking is on: less power emission

less area

reading curve in this range because of filters

The curves «after shifting» refer to the shift of the optical properties of the dye when the membrane voltage is modified by the spike.

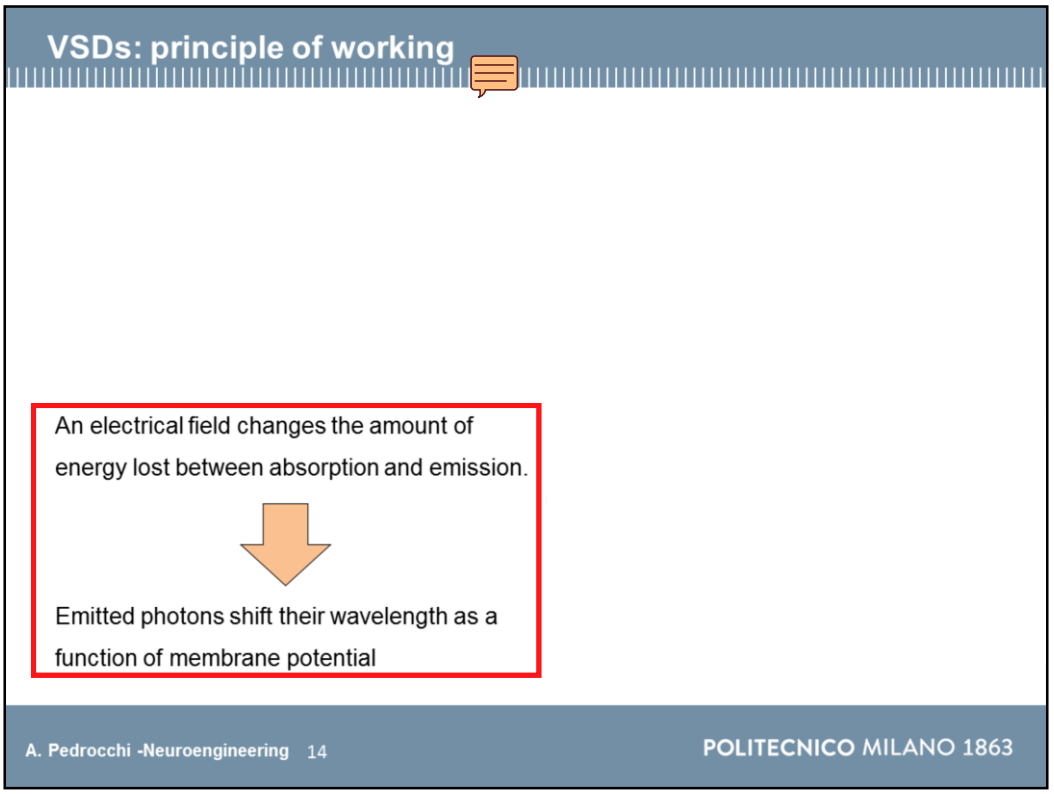
If the spike is on, there is a double effect.

First, a left shift of the excitation curve (red dashed curve), which results in a low absortion at the defined wavelenght (530nm). The low absorbtion by itself implies the reduction of the excitation light (blue dashed curve).

Further, the spike event induces a second mechanism which is a shift of the excitation curve (dot-dashed blue curve).

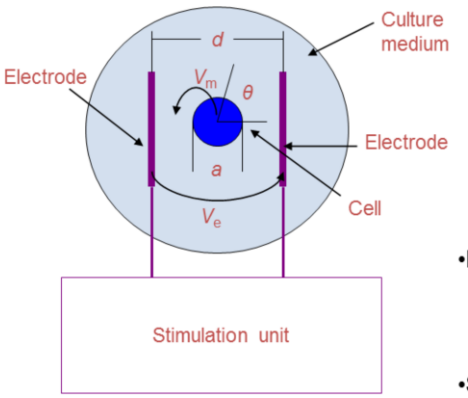
The design of the microscope is then optimized to maximize the difference between the emitted light in presence and in abscence of spike...

We can use fluorescence to know the calcium movement, but we could use it to know the neuronal activity (which is our goal btw, we use optical tools for recording neuronal activity) we need a specific fluorescent dye. Which is not only one, is a family, and they all goes under the name of VOLTAGE SENSITIVE DYES. VSD.
Their fluroescent properties change depending on the voltage! and neuoronal activity is encoded in voltage.
How do they work?
As soon as we put the filters we are reading from like a small area, not the curve
we have two effects happening together
--->**the compound is binding to the membrane of the neuron**, **if the voltage, across the membrane is depolarized (the spike is on) the excitation curve is moved to the dashed one.**
Emission: 1st effect: if we are reducing the power of excitation (bc red is shifted, due to change in voltage) we have less intanse, just scaling one.
2nd effect) Then we have a shift also on the emission curve to the left, which is increasing the difference between the excited to the respect the rest situation.



[43.00] You can check for the functioning of the molecules by applying an external stimulation to the neurons and see if you see in the image the different values of the emitted light. We expect that by applying an external electrode, we will have one side positve,and the other negative (of the perfectly circular neuron).

Experimental validation



Experimental validation has been carried out by electrical stimuli delivered through electrodes immerged in culture medium.

Culture medium is rich in ions: a flux of charges starts when $V_e \neq 0$.

lati esterni

The buildup of ions on cell membrane outer side causes a voltage gap between inside and outside of the cell.

•Electrical field between electrodes:

$$E = \frac{\Delta V_e}{d}$$

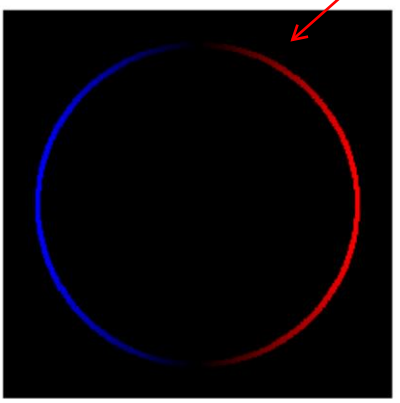
•Stimulation electrodes distance(*d*): 6,5mm

•Estimated cell diameter(*a*): 10µm

no

Experimental model

our expectation (perfectly round neuron)



In RED, expected negative fluorescence variation, in BLUE positive variation. False color diagram of expected membrane voltage.

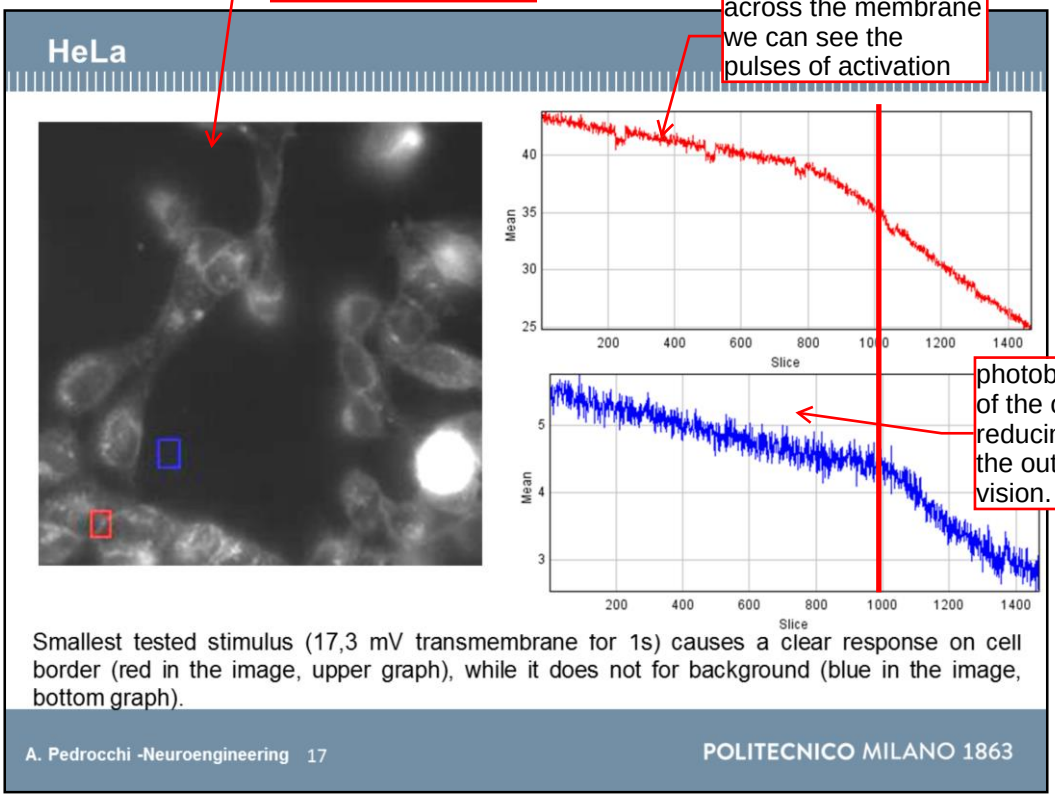
The imposition of an external electrical field causes voltage gap across cell membranes.

In the hypothesis of:

- circular ,
- separated,
- ion-proof cells,

what we actually get. HeLa: very standard excitable cell, which can be bought.

across the membrane we can see the pulses of activation



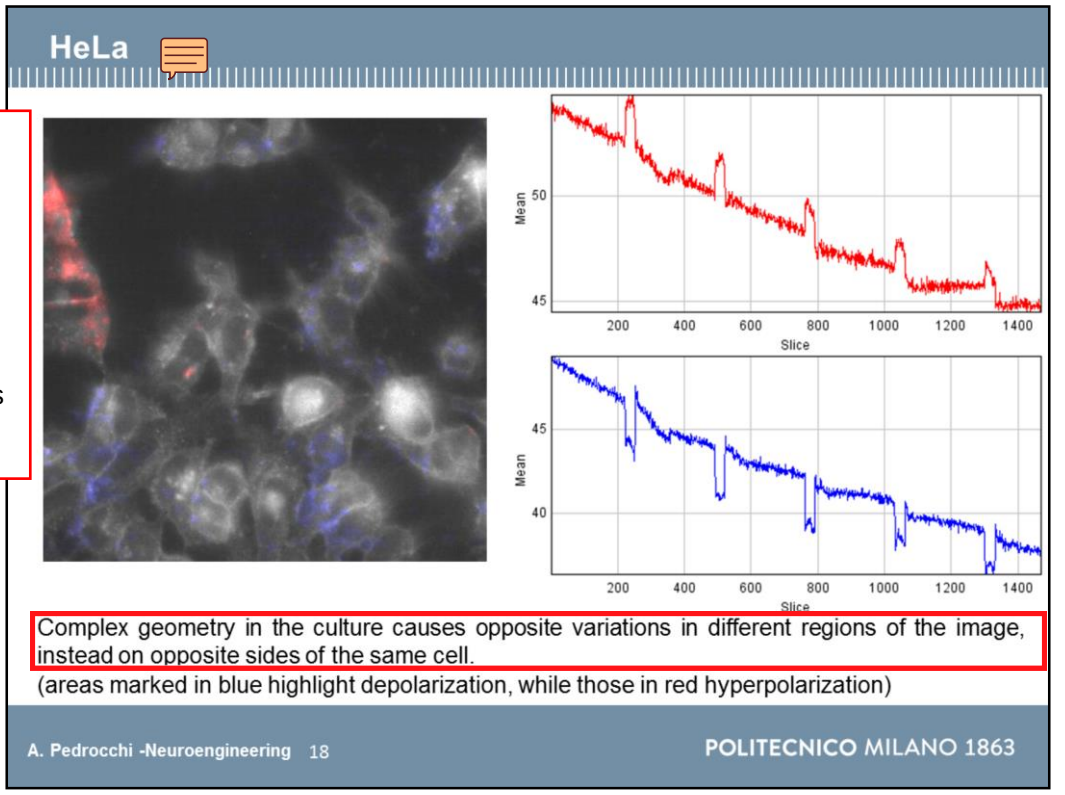
photobleaching: effect of the overall reducing over time of the output of the vision.

We can add a VSD to an HeLa culture, and we'll see that in the medium we just see the photobleaching, while across the membrane we can see the pulses of activation.

More dense area of the culture we'd see that, the cells facing the negative electrode, in respect to the ones facing the positive one, do show an opposite sign of the change of the emission. **This proves that dep. and high. polarization can be measured by this fluorescent dye.**

VSD vs MEA:
+ Spatial resolution
- Temporal resolution

VSD vs Patchclamp:
+ Easier
+ More nuerons
+ less invasive



V sopra

Major advantages of this to respect to MEA:

Advantages to patch clamp: not invasive, many neurons, easier.

To respect to MEA:
- we had the problem of spatial resolution. With images we can couple the activity with the image. So the spatial resolution will depend on the pixel size and objective of the camera (lenses).

VSD: high resolution if we use high sensor resolution, and short objectives. We can modulate the spatial resolution depending on the objective of the measurements by changing the cameras. We could be able to see the single part of the soma. we can focus on the single part of the soma, or we could want to have an image of the whole culture. And we could switch from one to the other.

And in the end you'll always have to compromise between spatial resoution and FOV. But, with standard ccd and standard lenses we can have the image of the soma and a FOV including the network of the whole petri, or a bit less.

Major limitation: problem of temporal resolution. In the case of MEA it can be as high as you want, you can acquire at 10khz, 1ms duration, who cares. You easily record spikes. Here you may have to chose specific camera to get the temperal resolution of the "single spot".
The fasted ccd camerae goes to 200Hhz, 500hz... maybe faster, like 1khz but with binding of pixels (like 4 pixels become 1). You are making a balance between spatial and temporal res. (ccd camera)

While, cmos camera, costs much more, but actually the most recent goes to 2khz without major problems and without binding.

Soo better spatial rese. in VSD to resepcet to mea, depending on the camera and objectives that we select, but this needs to be compromise with a temporal resolution.