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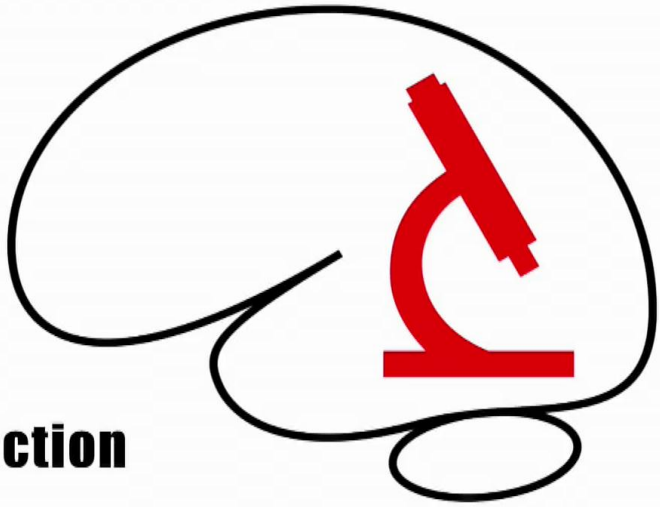
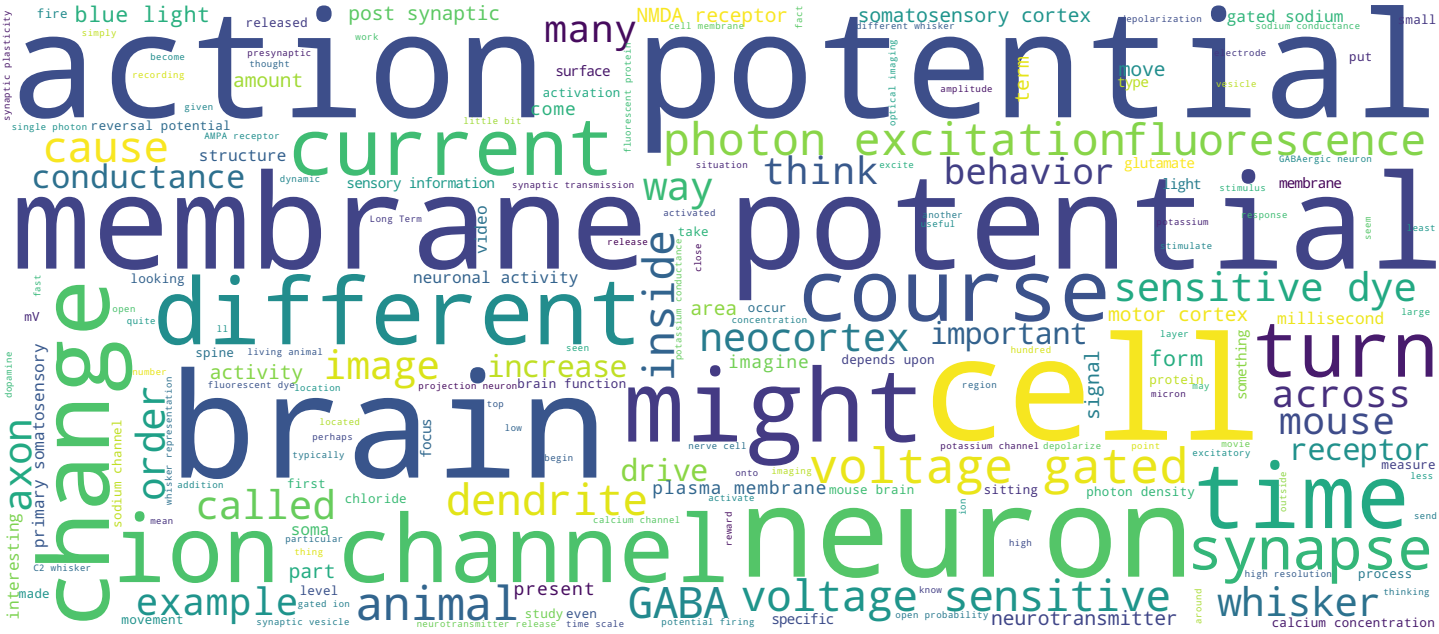


6.3 Imaging the brain in action

Cellular Mechanisms of Brain Function

Prof. Carl Petersen



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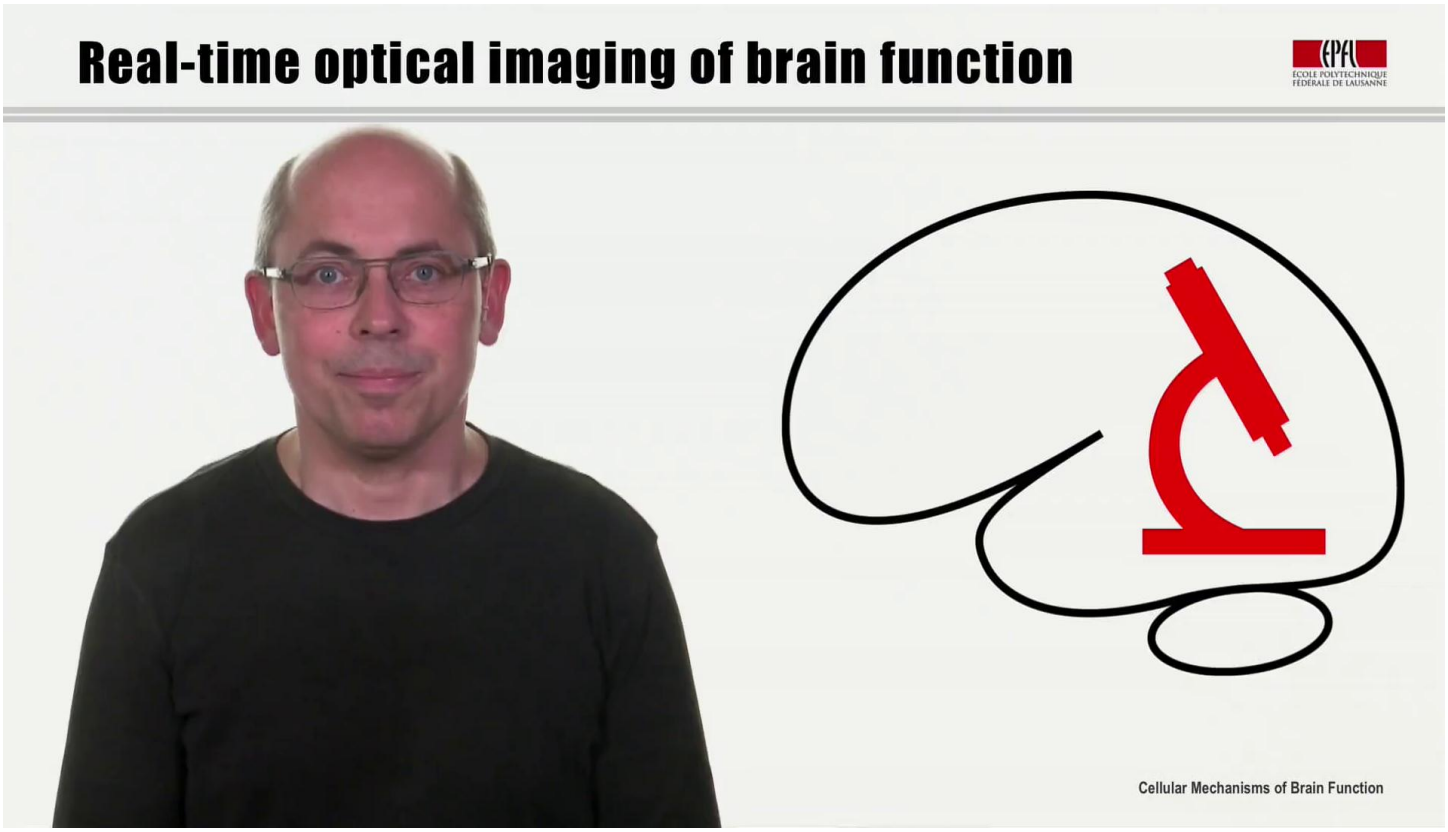
Video

EPFL



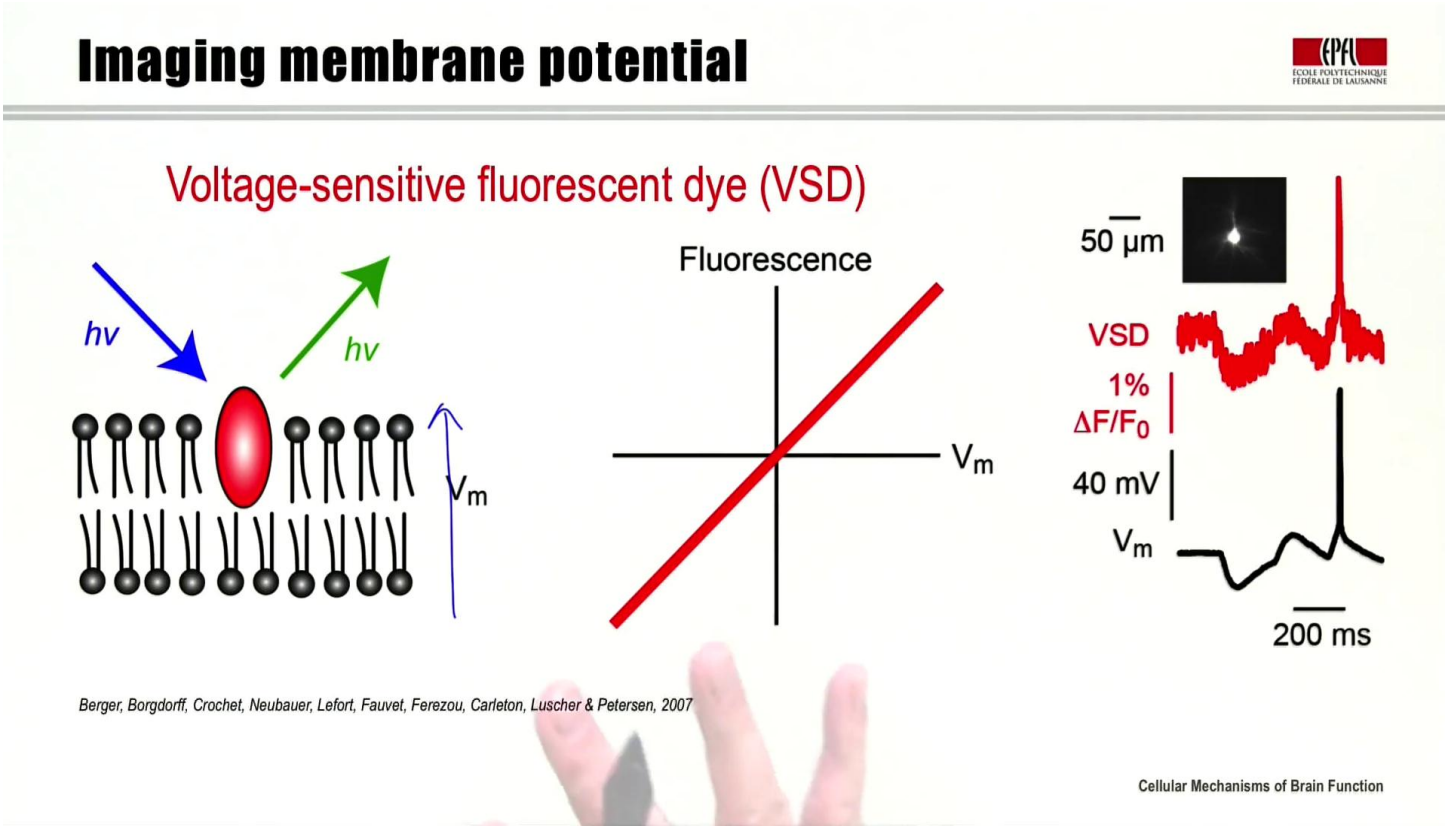
Our first step towards a causal and mechanistic understanding of how brain function drives behavior is to measure neuronal activity at high resolution, and to correlate that with the ongoing behavior of the animal. In this video, we're going to see how imaging approaches are useful at defining the spatial-temporal dynamics of brain function. Optical imaging approaches turn out to be particularly useful for high-resolution imaging of what's going on in the brain during behavior. In this video we'll explore two different fluorescence imaging approaches, one which gives large fields of view, and can define neuronal activity at the level of hundreds of microns, at millisecond temporal resolution, and another one where we can zoom in and look at higher resolution, seeing individual nerve cells with micron and submicron resolution, but with smaller fields of view.

Notes

Summary

0m 05s

Imaging membrane potential



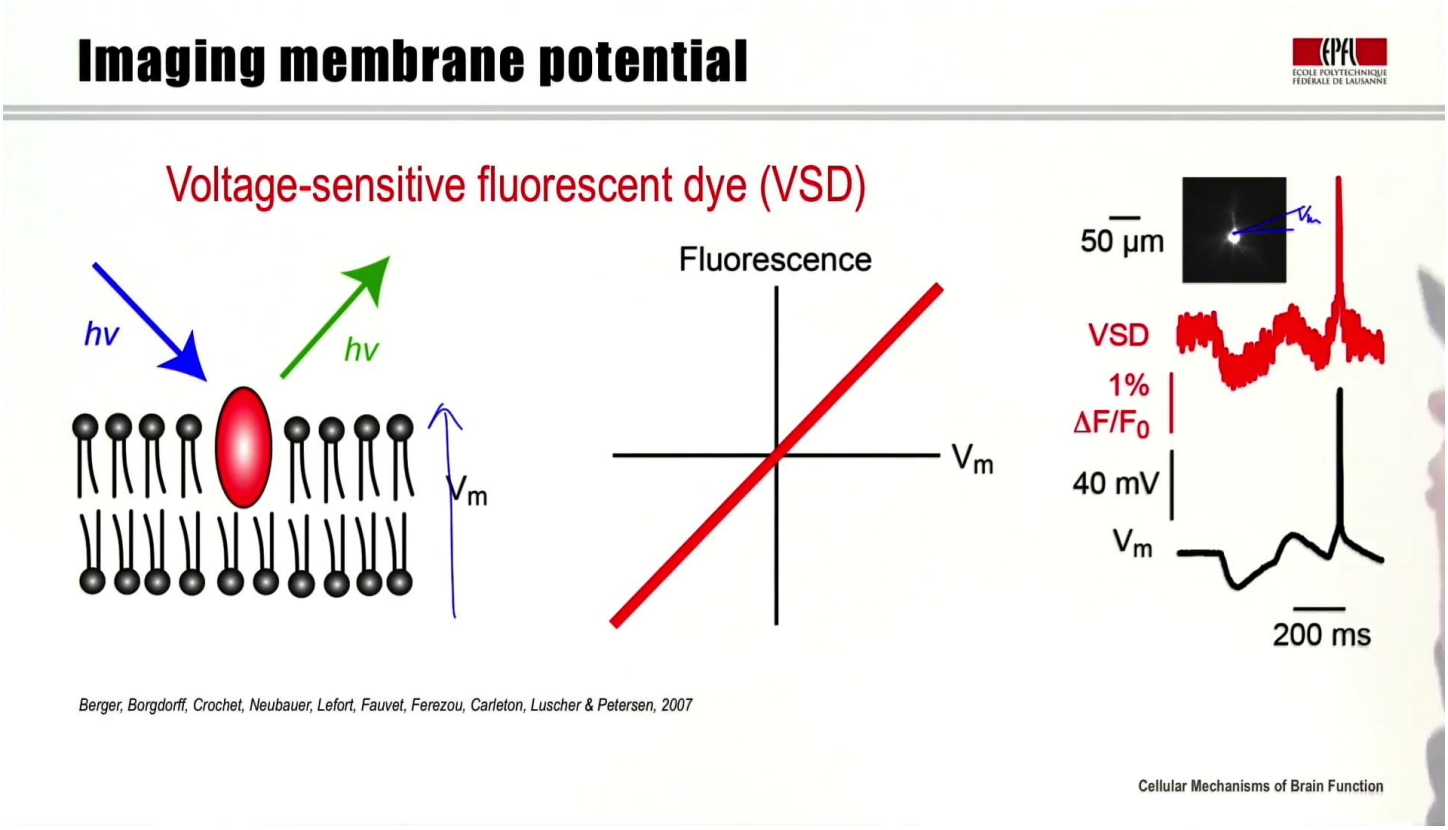
Over the past weeks, we've discussed how the brain is largely an electrical signaling device. And so if we want to image neuronal function, a good starting point is to think about how we might image membrane potential. Scientists have worked towards developing voltage-sensitive fluorescent dyes for many years, and some of these have become very important and useful. The voltage-sensitive dye is typically a lipophilic compound that can be added either to the inside, or outside of neurons. It inserts into the lipophilic cell membrane, it's a fluorescent molecule, and so we can put in, say, blue photons to excite, and longer wavelength, say green photons, are emitted in a standard fluorescence process. For a voltage-sensitive fluorescent dye, the fluorescence depends upon the membrane potential. So the electric field, across the plasma membrane, drives the so-called electrochromic effect, and the fluorescence then depends upon membrane potential. And for a typical voltage-sensitive dye, the fluorescence is linearly correlated with membrane potential. Many fluorescent dyes are responsive to membrane potential on the microsecond timescale, and so the changes in fluorescence correlate exactly with the change in membrane potential at the timescale of neuronal activity.

Notes

Summary

1m 04s

Imaging membrane potential



Here's an example of voltage-sensitive dye imaging from an individual neuron, located in a brain slice in vitro. The neuron has been filled with voltage-sensitive dye from the inside, so this dye is now sitting on the inner leaflet of the plasma membrane. You see the soma. You probably also see some of the finer processes, the dendrites, that emerge from the soma. At the same time that we're imaging this neuron, we also have an electrode here that's recording the membrane potential of that cell. And through the patch clamp electrode we can also change the membrane potential by injecting different currents. And so initially here we can hyperpolarize the membrane potential, we can depolarize the membrane potential, and by injecting a bit more current, we can fire an action potential. And so that's our standard membrane potential measuring technique that we've been looking at over the last weeks. Now we've also got the voltage-sensitive fluorescent dye where we can measure the fluorescence from this neuron at the same time that we measure the membrane potential.

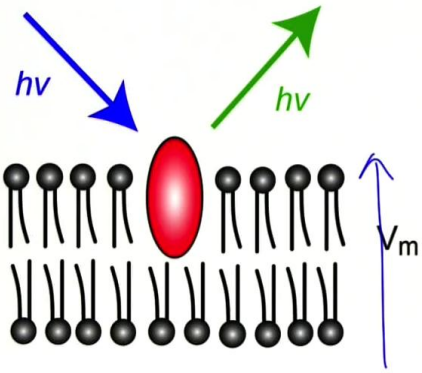
Notes

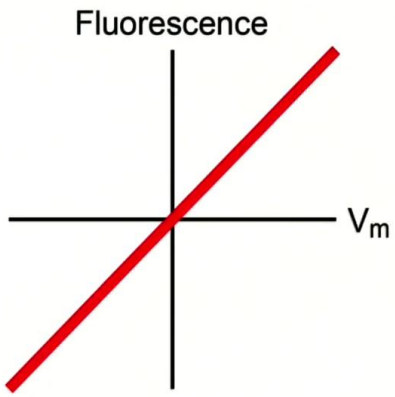
Summary

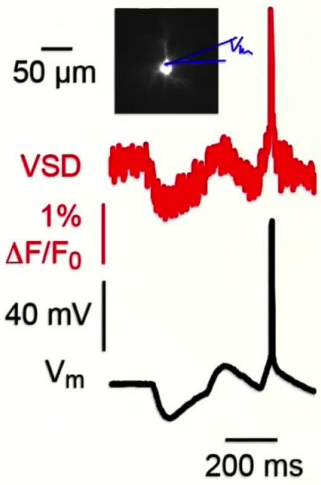
2m 37s

Imaging membrane potential

Voltage-sensitive fluorescent dye (VSD)







Berger, Borgdorff, Crochet, Neubauer, Lefort, Fauvet, Ferezou, Carleton, Luscher & Petersen, 2007

Cellular Mechanisms of Brain Function

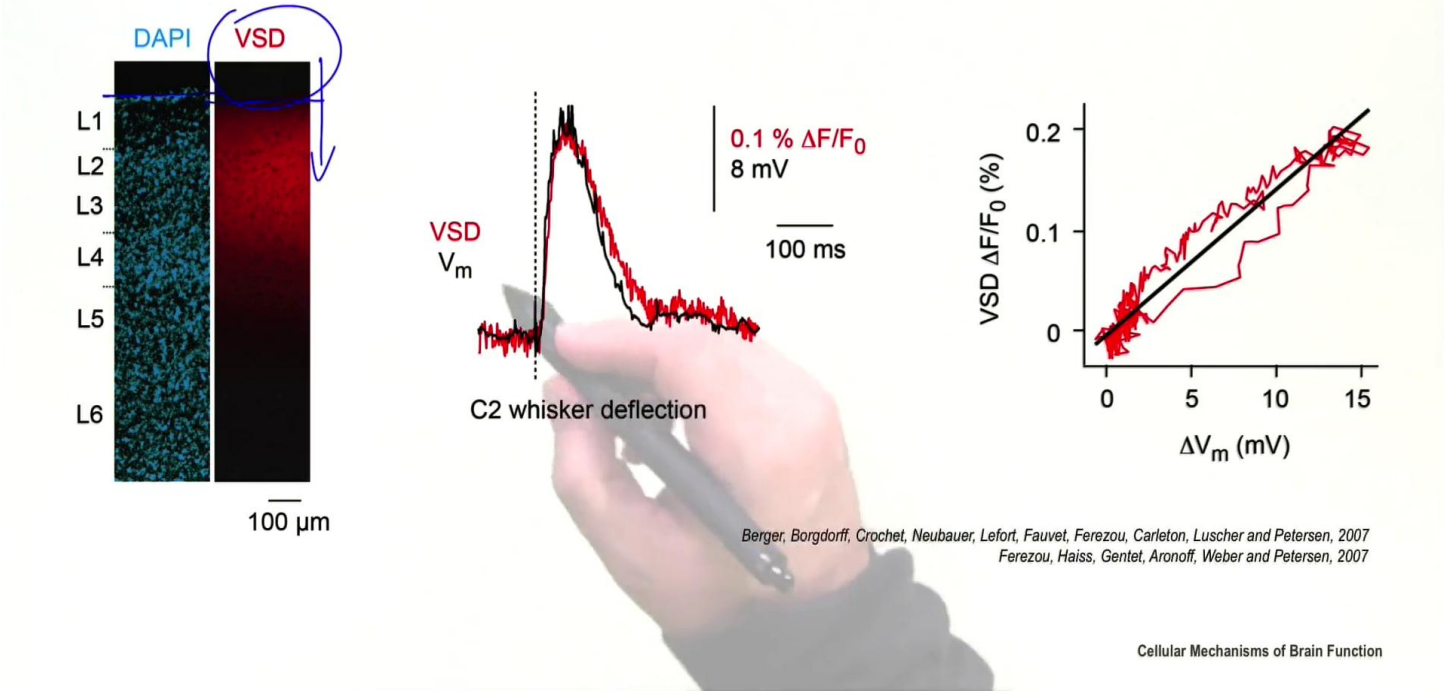
And you can see that as we hyperpolarize a cell, the fluorescence decreases, as we depolarize a cell, the fluorescence increases again, and as we fire an action potential in that cell, we also see a rapid transient in the fluorescence of the voltage-sensitive dye. So here you can see an optical method for measuring membrane potential in individual nerve cells in a brain slice. The voltage-sensitive dye is noisy, the signals are small, and so far it's not been possible to make these measurements inside the living animal in vivo.

Notes

Summary

3m 42s

Imaging membrane potential *in vivo*



Berger, Borgdorff, Crochet, Neubauer, Lefort, Fauvet, Ferezou, Carleton, Luscher and Petersen, 2007
Ferezou, Haiss, Gentet, Aronoff, Weber and Petersen, 2007

Cellular Mechanisms of Brain Function

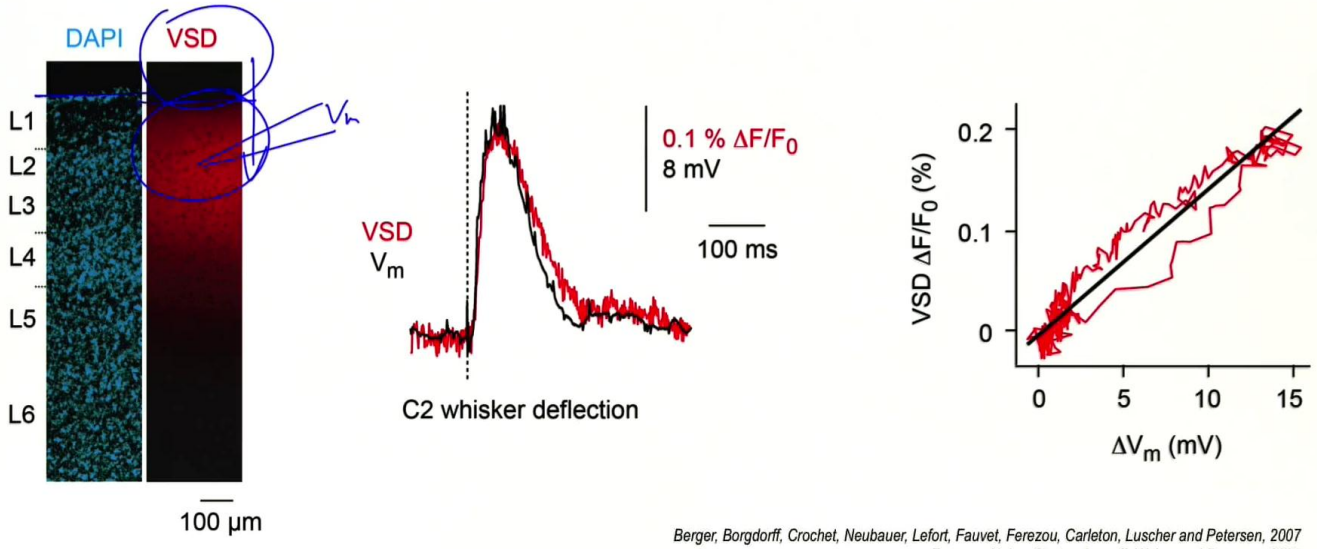
However, the voltage-sensitive dye imaging technique has been applied at the level of populations of neurons, where we can stain many neurons from the outside, by applying the voltage-sensitive dye from the top of the brain, allowing it to diffuse inside the brain simply through the extracellular spaces, the surface of the brain. We have to remove the bone, we apply the dye to the surface, it diffuses in, and maybe it diffuses in the top half of the neocortex, so this is the full extent of the neocortex here, visualized with DAPI to see where the cell bodies are, where the DNA is, of the nuclei of different cells, and the voltage-sensitive dye stains roughly the upper half, 500 microns or so, of the surface of the neocortex. If we now look from the top of the brain and image this fluorescence in a living animal, and we apply a sensory stimulation like, for example, a whisker deflection, that then drives a depolarizing sensory response in the somatosensory cortex. We can measure that with a membrane potential recording, so we can measure the membrane potential from a cell.

Notes

Summary

4m 18s

Imaging membrane potential *in vivo*



Berger, Borgdorff, Crochet, Neubauer, Lefort, Fauvet, Ferezou, Carleton, Luscher and Petersen, 2007
Ferezou, Haiss, Gentet, Aronoff, Weber and Petersen, 2007

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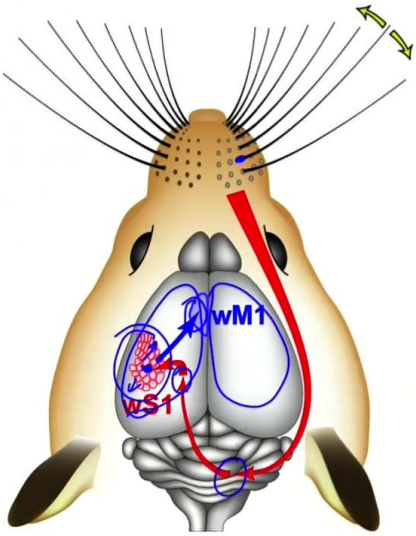
We deflect the whisker, that causes a depolarization in the neuron, and if simultaneously we measure the fluorescence from this area of the brain, we see that there's a very similar time course to the fluorescent changes, and we can again plot linear fluorescence, versus membrane potential traces, for the in vivo situation. So here we have an optical method for measuring membrane potential from neuronal populations at the millisecond timescale, and this then allows us to examine the spatial-temporal dynamics of cortical function in living animals.

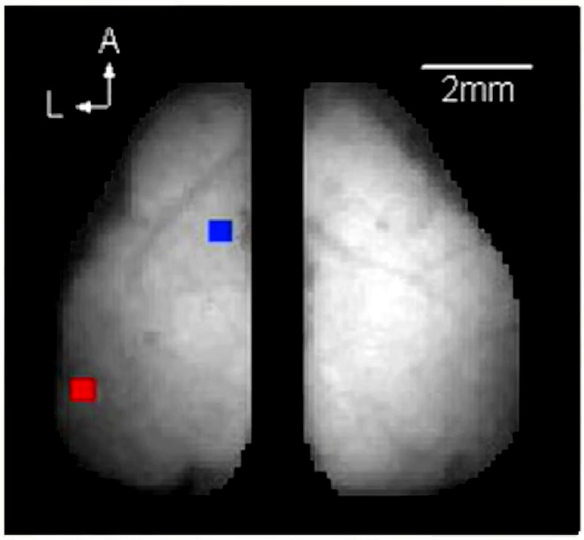
Notes

Summary

5m 30s

Spatiotemporal dynamics of cortical function





Ferezou, Haiss, Gentet, Aronoff, Weber and Petersen, 2007

Cellular Mechanisms of Brain Function

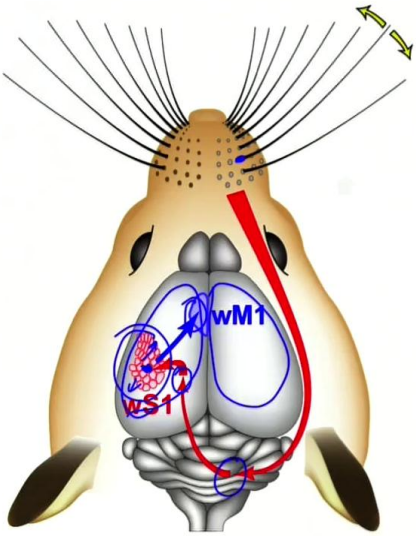
And so the experiment that I'd like to show you is one in which we've opened up craniotomies across a relatively large part of the dorsal surface of the mouse brain, that's what you see here. This is a craniotomy, we put voltage-sensitive dye on top of the brain, that then causes it to be fluorescence, and what you see here is the fluorescence from the left and the right hemispheres of the mouse brain. In the movie that we're going to see in a second, we deflect the whisker, that then causes a signaling pathway to be activated through the trigeminal nerve. So we give a one millisecond deflection of the whisker, we get a one millisecond volley of action potential down the trigeminal nerve, glutamate was released in the brain stem, activates postsynaptic neurons that again fire a brief volley of action potentials that are then transmitted to the thalamus. Again, glutamatergic synaptic transmission takes place, and that then brings the sensory information to the neocortex, where we have a tight localization here of the C2 whisker to a small, localized region of the primary somatosensory cortex, the S1. And what we'll see in the movie is that the information doesn't remain localized to this area of the brain, but rather spreads over quite a large area, activating the nearby somatosensory cortex, and also the motor cortex area of the mouse brain.

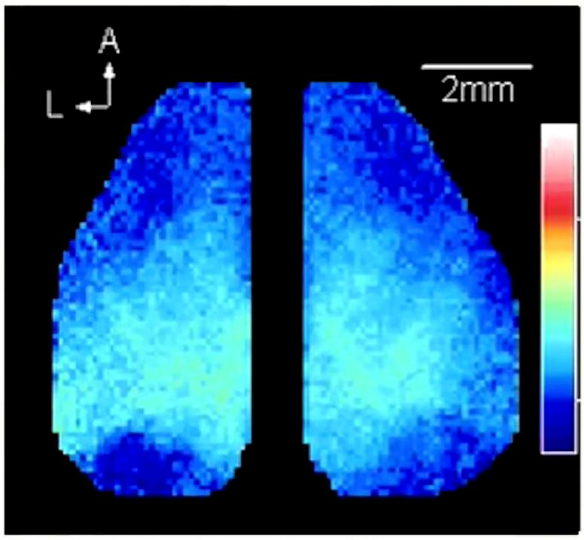
Notes

Summary

6m 06s

Spatiotemporal dynamics of cortical function





Ferezou, Haiss, Gentet, Aronoff, Weber and Petersen, 2007

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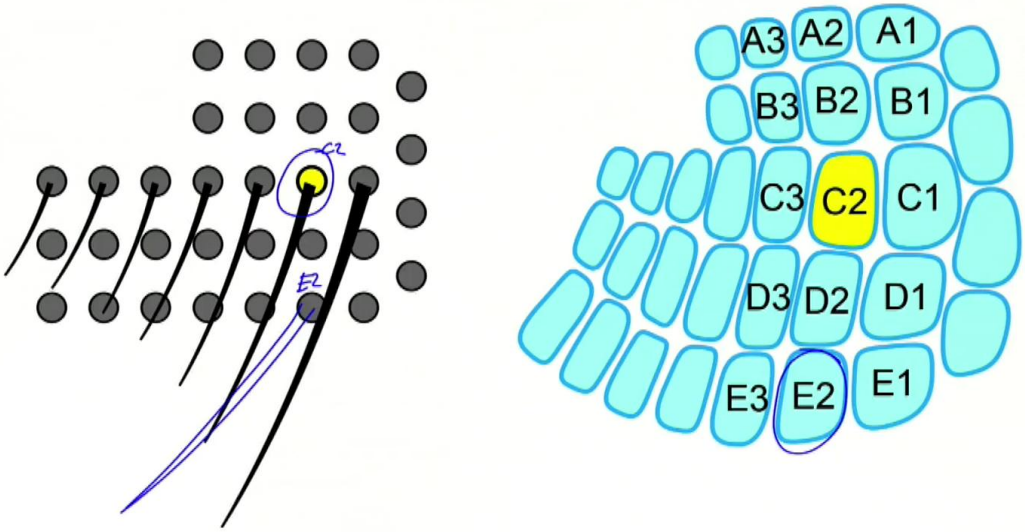
So let's have a look at the movie. So the stimulus is given. A few milliseconds later, we get a localized hot spot. It spreads across the primary somatosensory cortex, and then activates this frontal area of the motor cortex. And at one level, the spread of sensory information from that highly localized initiation site makes good sense, in terms of thinking about how an animal would be exploring its environment. It has many whiskers, and the way that it'll detect the size and shape of an object is by comparing the sensory information coming from the different whiskers contacting that object. And so it's essential that sensory information spreads from one whisker representation to its neighbor's. And so that also explains the local spread of information across primary somatosensory cortex. It also makes sense that the information spreads to the motor cortex. We expect our movements to be informed by ongoing sensory input. And so what we have here then, is a visualization of a sensory motor loop, where sensory information comes in, it gets processed, and then passed on to the motor cortex to drive the next steps of the behavior of the animal.

Notes

Summary

7m 34s

Somatotopic whisker map



Cellular Mechanisms of Brain Function

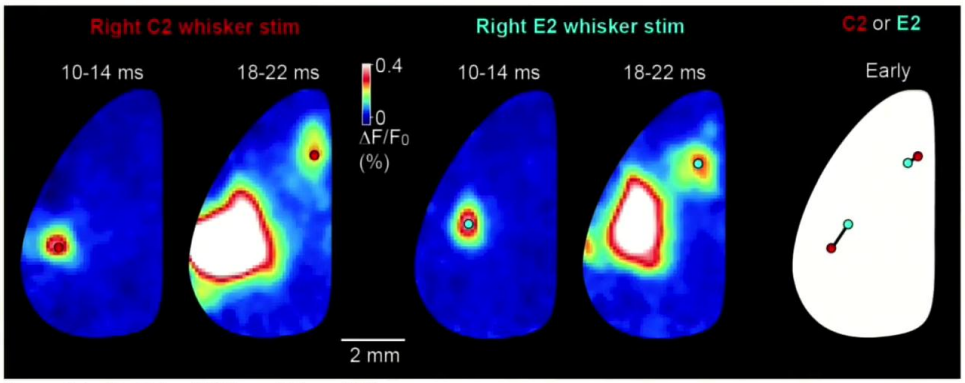
Now, there's a highly organized somatotopic map. In the primary somatosensory cortex that we've already considered the layout of the whiskers is precisely mirrored by the layout of the so-called barrel columns in the primary somatosensory cortex. So the C2 whisker representation is mapped onto the C2 barrel column, and the E2 whisker, that's located here, maps onto the E2 barrel column representation and primary somatosensory cortex. And so by stimulating different whiskers on the snout of the animal, we'd also expect that the initial localization of the sensory response should also move around in a somatotopic manner on the surface of the cortex. And that's exactly what we can see with voltage-sensitive dyes.

Notes

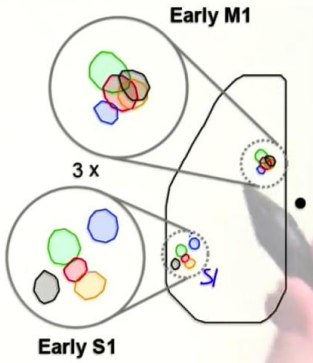
Summary

8m 47s

Mapping mouse sensorimotor cortex



Ferezou, Haiss, Gentet, Aronoff, Weber and Petersen, 2007



Cellular Mechanisms of Brain Function

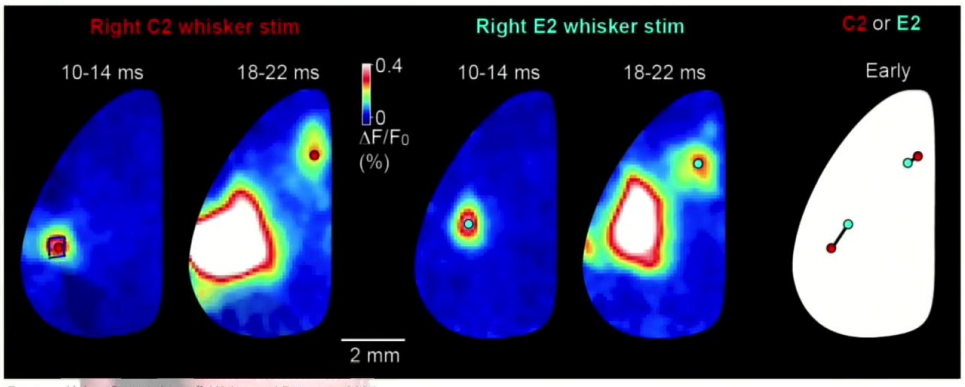
Here on the left, we deflect the C2 whisker, we get an early, localized sensory response, some ten milliseconds after whisker deflection. If instead we deflect the E2 whisker, we see that these also are localized sensory reponse, but it's moved, relative to the C2 whisker representation. It's moved by roughly 1 mm on the surface of the brain. Of course there's a spread of the sensory information within the next milliseconds after the initiation. That's what we saw in the video, and we also see the activation of this frontal area, the whisker motor cortex. Interestingly, there's also a sensory map here in this frontal motor cortex area, so when we stimulate the C2 whisker, we activate one epicenter, and the E2 has a slightly shifted representation of its whisker. So there's an interesting change in both the map in sensory cortex, and also the sensory map in motor cortex. And we can stimulate different whiskers, and you can see that there's a large gap between different whisker representations in S1. And if we move to motor cortex, these different whiskers are represented in a highly compressed map. And in addition there seems to be mirror symmetry, where the E2 whisker is represented close to the line of symmetry, and the A2 whisker is relatively far apart.

Notes

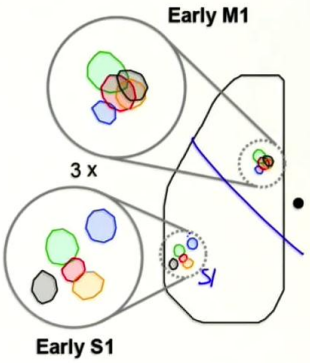
Summary

9m 37s

Mapping mouse sensorimotor cortex



Ferezou, Haiss, Gentet, Aronoff, Weber and Petersen, 2007



Cellular Mechanisms of Brain Function

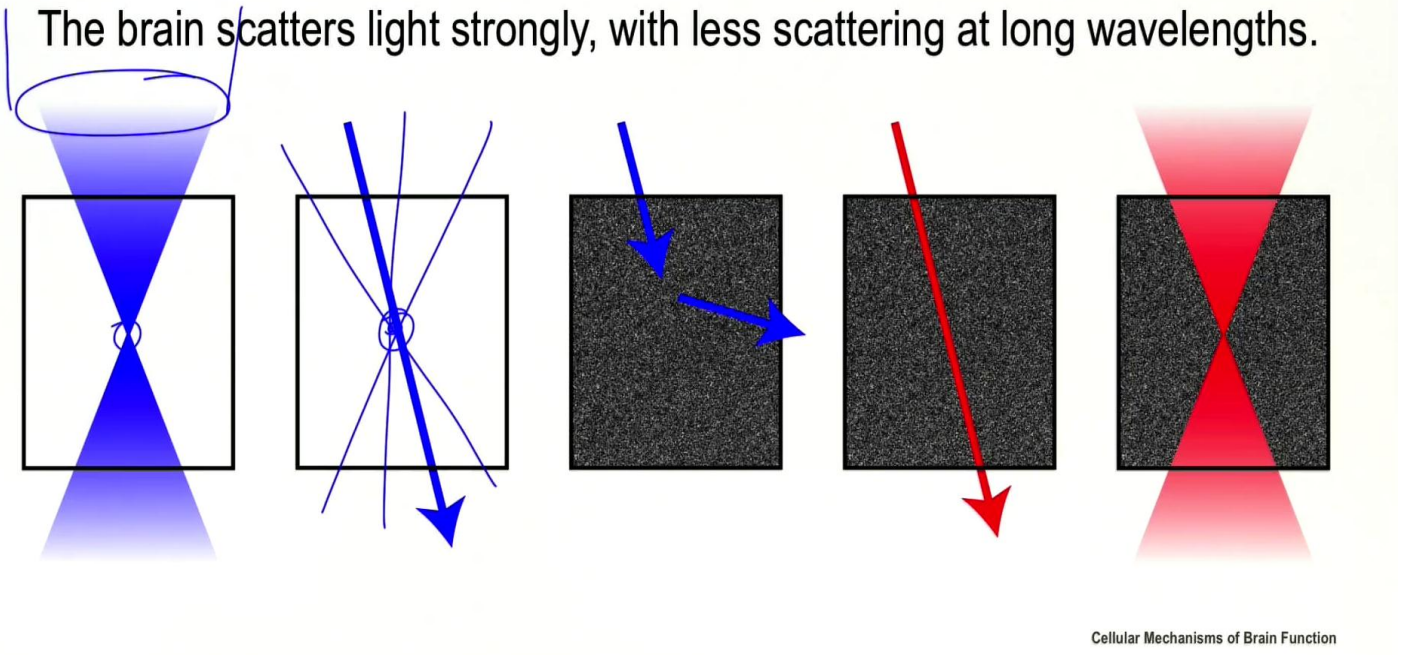
And so we can begin to build up functional maps of both sensory and motor areas of the mouse neocortex by using voltage-sensitive dye imaging in vivo, in awake behaving animals. Now, we would also like to be able to zoom in and look at higher resolution, at this particular area here of the brain, and study in more detail what's happening with cells, neurons, and synapses.

Notes

Summary

10m 59s

High resolution optical imaging



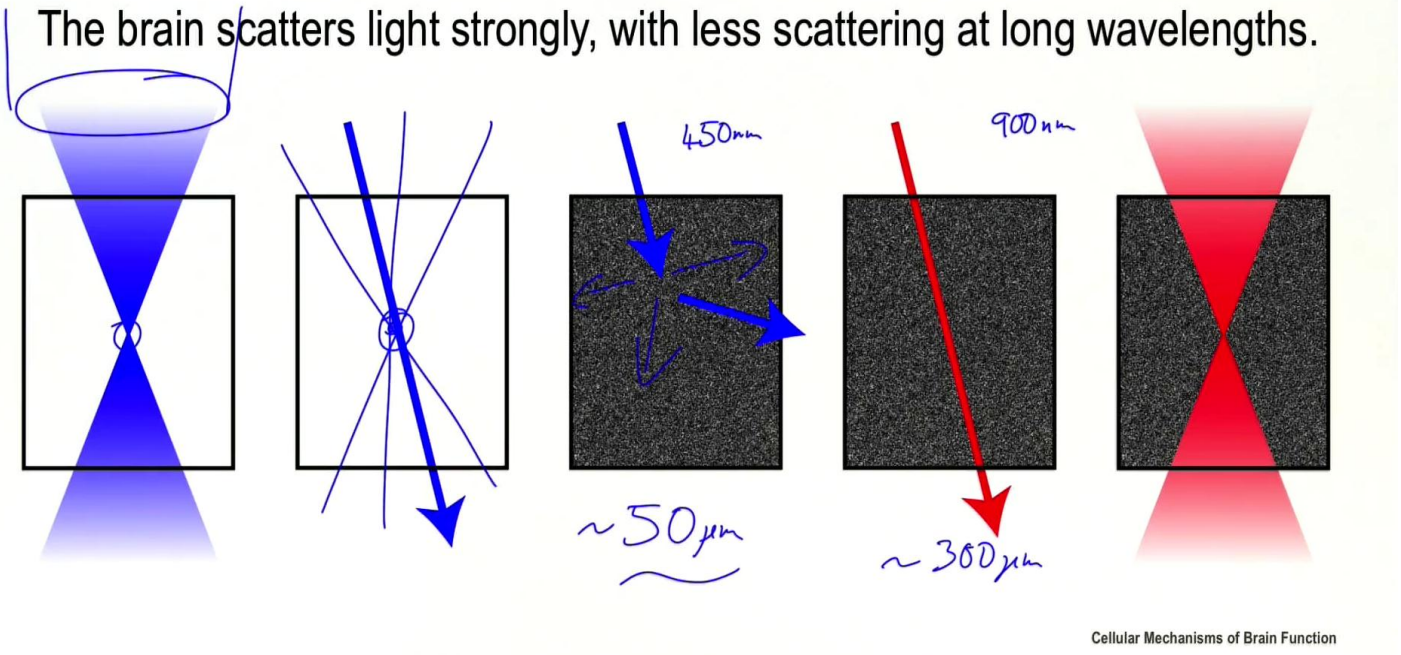
And in order to do high-resolution optical imaging, we need to face one further major problem with imaging in the brain, and that is that the brain is a highly scattering medium. And so if we imagine our normal microscope optics, where we have a microscope objective and we send blue light, say, into a non-scattering medium, we can focus that light down to a small spot, here, a diffraction-limited spot that depends upon wavelength and numerical aperture of the optics. And the reason we can do that is because the photons go through, more or less, without scattering through this medium, and so we can send in photons, all of which will intersect at the focal point of the microscope. And that we can then use for exciting fluorescence at one nice localization spot in the sample. Now, the brain turns out to be highly scattering, and so if we send blue light into the brain, then within a distance on average of around 50 micrometers that blue light will get scattered, it will get deflected in a multitude of different directions, and it'll then become impossible to focus a light down to a small spot, which is required in order to achieve high-resolution imaging.

Notes

Summary

11m 27s

High resolution optical imaging

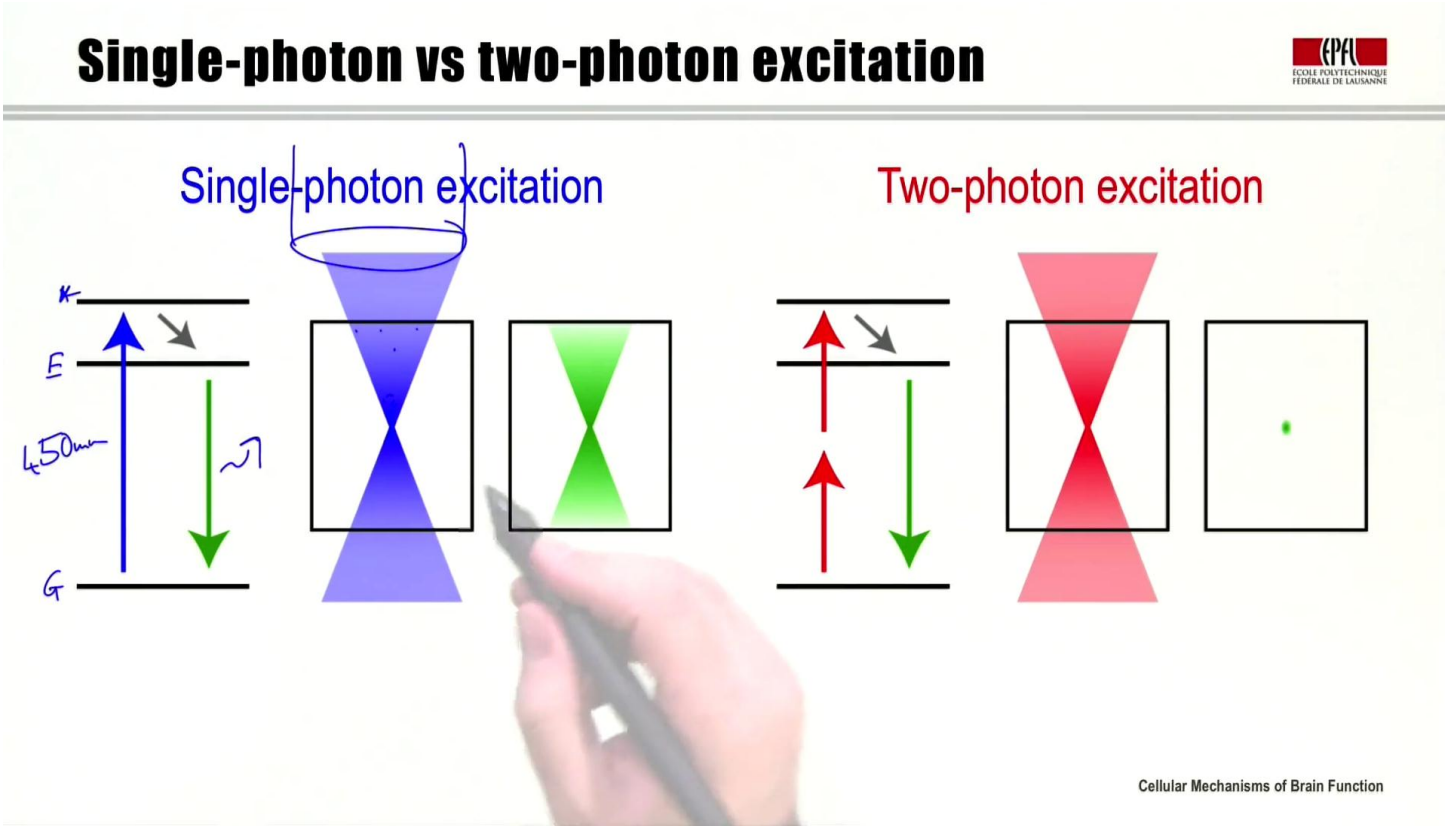


And so scattering is a major problem, in terms of imaging, in the brain. And if you want to image anything more than 50 micrometers from the surface of the brain, then it's extremely difficult to do it with standard one-photon excitation of fluorescence. The situation's a little bit better if you use longer wave-length light. And so if here we might have been using 450 nm blue light, we can instead use much longer wavelength for white light, maybe 900 nm in the near infrared. And at these longer wavelengths, the brain scatters much less, and it then becomes possible to focus a light at least better than it is for the short wavelength light, and so for infrared light of around 900 nm we have a mean path length of something like 300 microns, and that allows us then to look at least into the upper layers of the neocortex with higher resolution than with the short wavelength blue light.

Notes

Summary

12m 49s

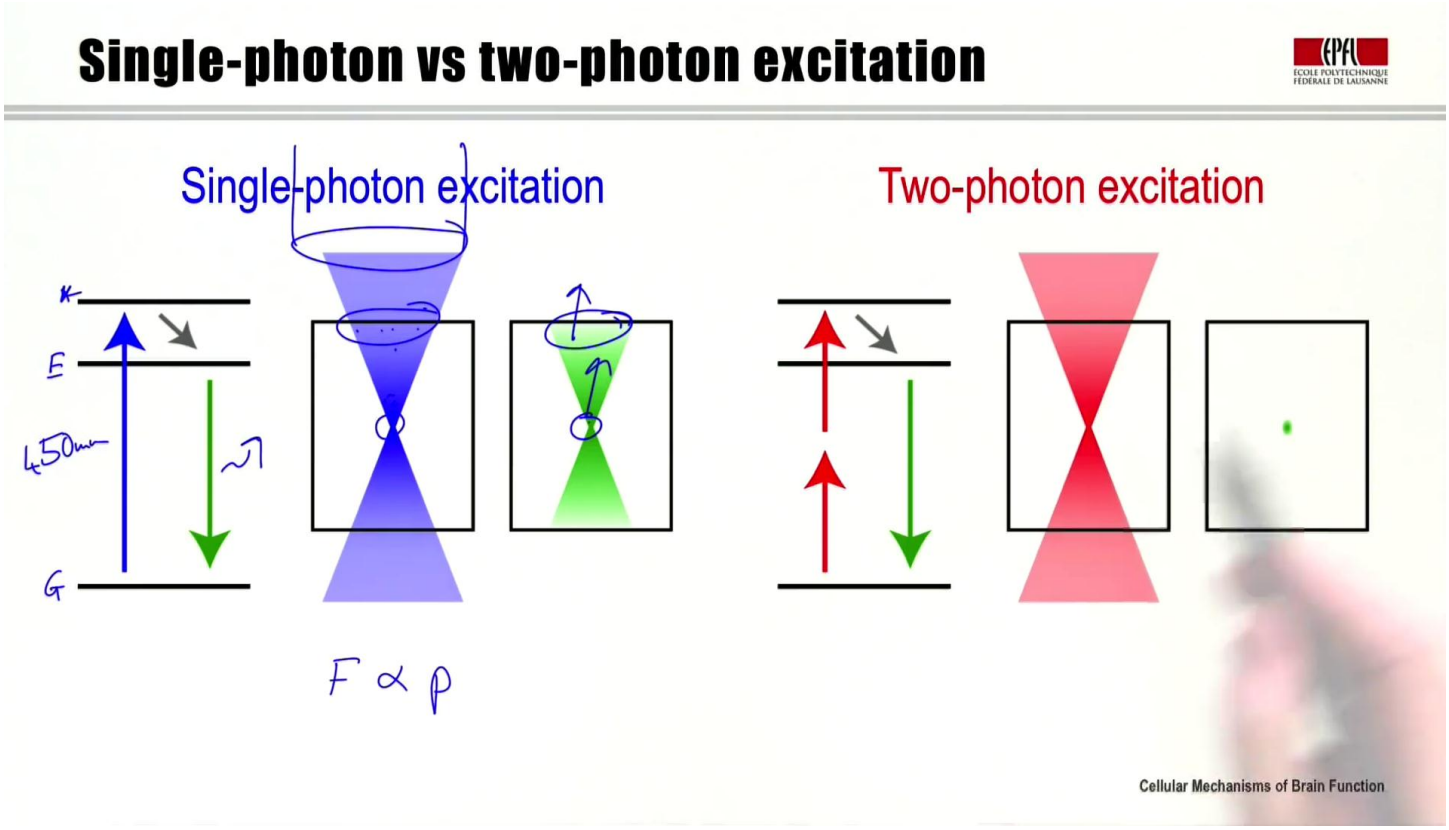


We have one further major problem in terms of imaging densely labeled fluorescing structures in the brain, and that's because of the way that single-photon excitation typically works. And so in standard fluorescence microscopy, we send in one high energy photon, say 450 nm blue light, that is used to excite the fluorophore, so this is a Jablonski-state diagram. We have the ground state, we have an excited state, and we have the initial excitation state, after absorption of the blue photon. There's then some rotational energy that's given off. It settles into an excited state. It remains there for some nanoseconds, and then emits the green fluorescent photon. And if we now imagine having a cuvette filled with a fluorescent dye in the solution, and we put blue light in, again, through our objective, we focus it in. Now, it's a non-scattering medium, so we focus a blue light in and the blue light then traverses this fluorescing medium. And of course the density of photons is low here, and it gets much higher around here. And so the amount of fluorescence in single-photon excitation is directly proportional to the photon density.

Notes

Summary

13m 54s

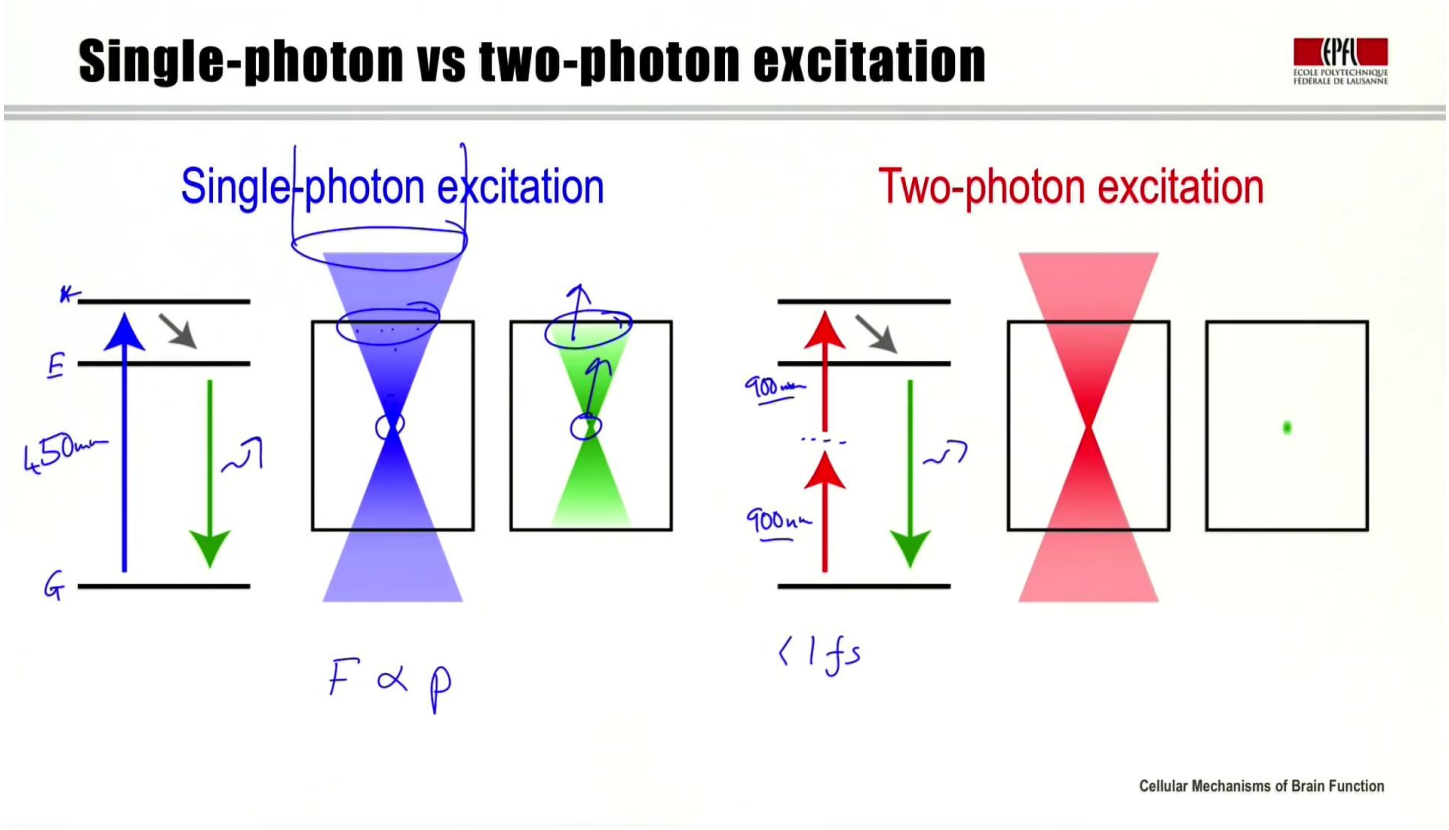


And so as we concentrate the photons here, we of course get more fluorescence here, emitted from the middle, where we have a high photon density, but the number of photons up here is quite considerable, and indeed every photon that ends up here, at the focal point, had to come through this cone of light. And so the total number of photons that are being absorbed and creating fluorescence here is just as high as it is here, it's just that here it's spread over a large area, and here it's highly concentrated. And so you can imagine that if we're trying to image into a densely fluorescent sample, the amount of fluorescence that comes from here will be, in fact, just as large as the amount of fluorescence from the focal point, here. And so in densely labeled structures it's impossible to get high-resolution imaging with single-photon excitation, because each photon is equally able to produce fluorescence in a single-photon excitation mode, and so the low density of photons here will create a lot of out-of-focus light, fluorescence. Two-photon excitation avoids many of these problems, by using a non-linear optical technique.

Notes

Summary

15m 20s

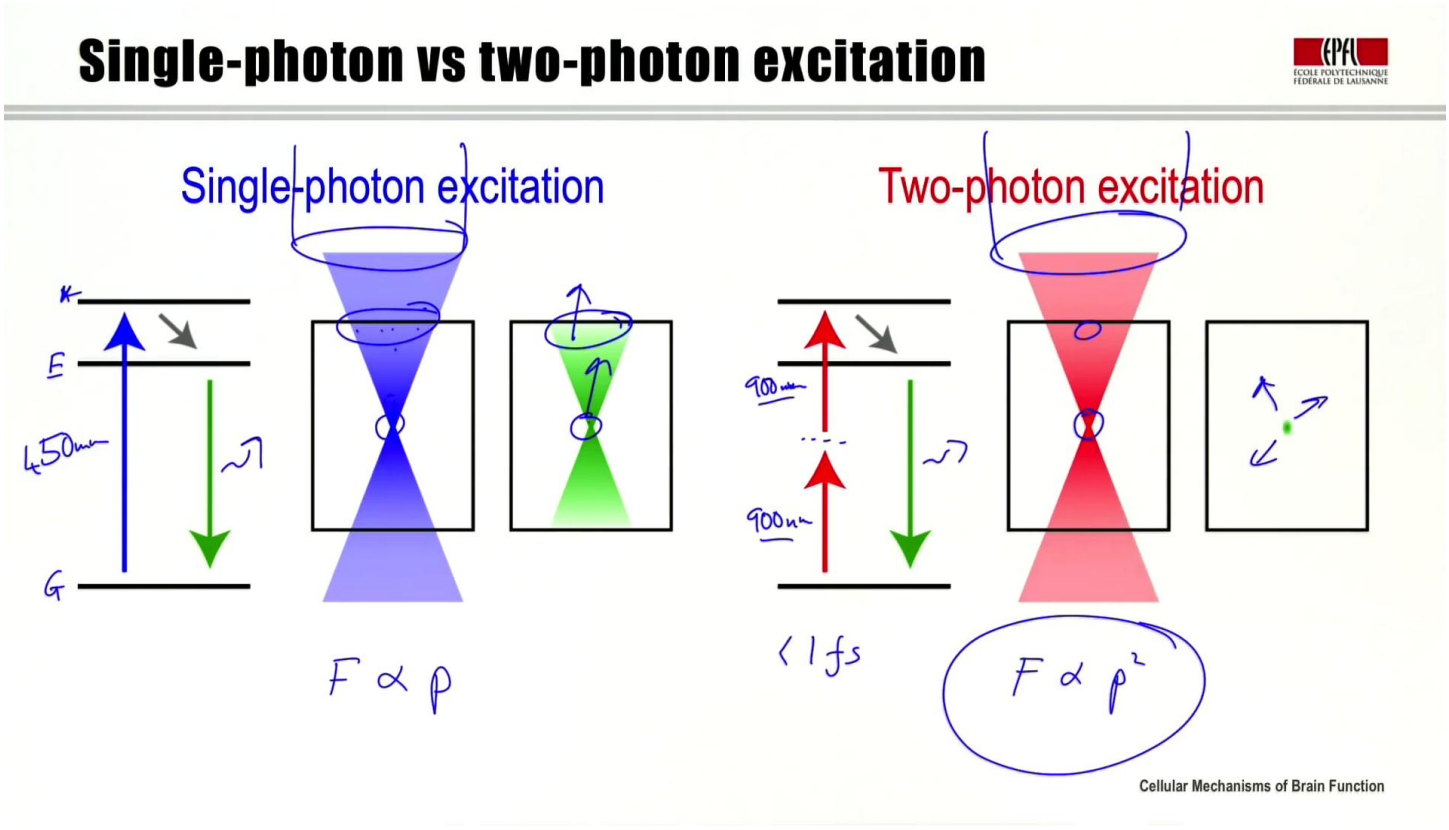


In order to excite the fluorophore, instead of taking one high-energy photon, as we do in single-photon excitation, we instead take two low-energy photons. And so we might, for example, have two 900nm photons that are absorbed, their energy here would be equivalent to the 450nm light, in the single-photon case. These two photons need to be absorbed nearly simultaneously. We go through a virtual state, here, in the Jablonski diagram. That virtual state has a lifetime of less than one femtosecond, so basically these two photons need to be grabbed simultaneously. That excites the fluorophore, and again we get the emission of our standard green fluorescence. Now, the first advantage with two-photon excitation, then, is that we're using longer wavelength photons to excite our fluorophores. And we already just mentioned that there's less scattering of long-wavelength light, and so it's easier to focus the light into the scattering medium of the brain. So that's one advantage of two-photon excitation, is that we start off with low-energy photons of long wavelength. But the real advantage of two-photon microscopy comes in the squaring of the photon density that's required to generate the two-photon excitation.

Notes

Summary

16m 36s



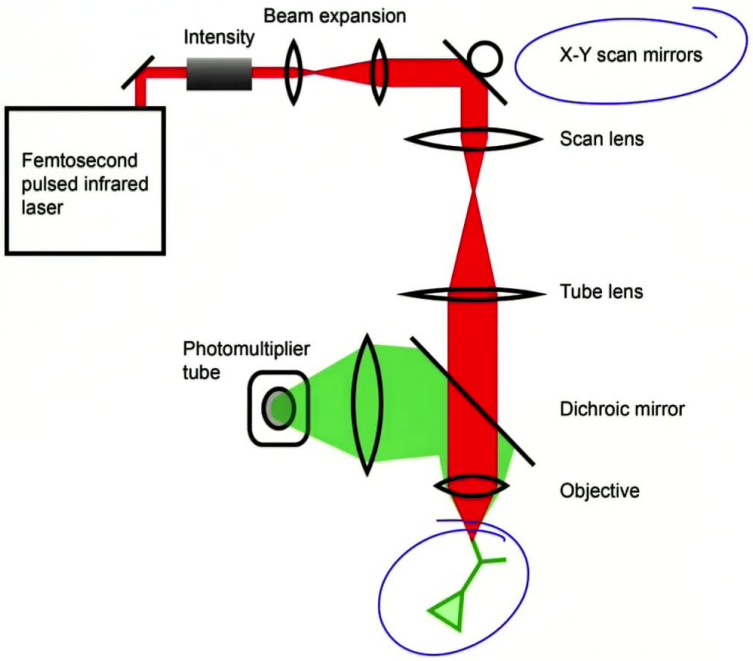
So because we need to absorb two photons near simultaneously, it turns out that the probability of generating this two-photon excitation depends upon the square of the photon density. It's like multiplying probabilities. The probability of grabbing one photon depends upon the photon density, and grabbing two simultaneously will then be the square of the photon density. And what that means, in terms of looking at the emitted excitation, the fluorescence light under the two-photon excitation, is that these areas, here, that have low photon density, will basically generate no fluorescence, whereas the high photon density that we get from the focal spot generates an intense fluorescence. And so we limit the excitation to a small focal volume in the two-photon excitation mode. And we then do not have any out-of-focus fluorescence, and all the emitted photons then come from this excitation volume through two-photon excitation.

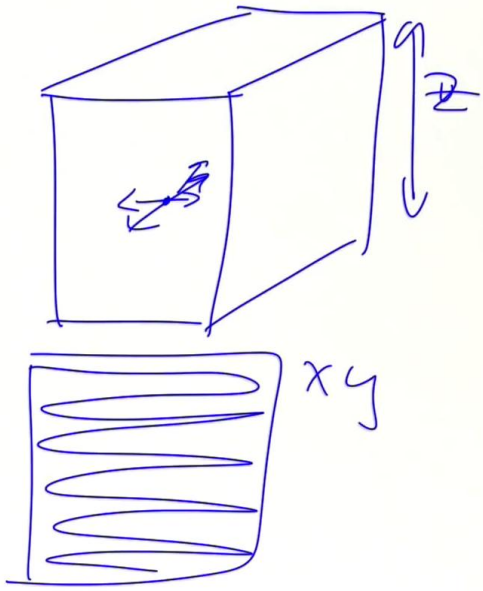
Notes

Summary

18m 03s

Two-photon imaging





Cellular Mechanisms of Brain Function

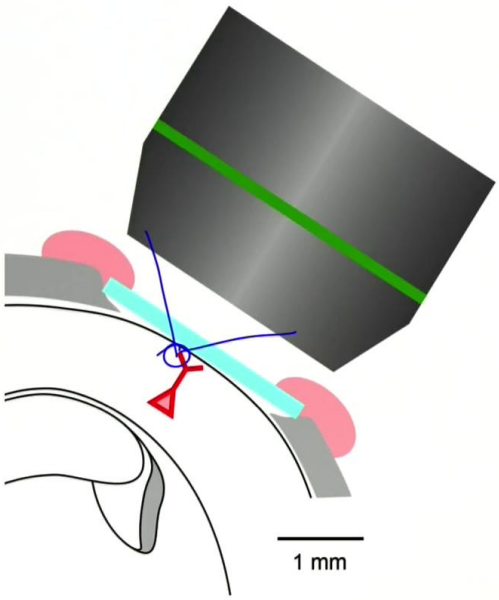
We can then use this small volume, that gets excited by the two-photon excitation, to image what's going on in the brain. Now, we only have a small volume that's getting excited, and so if you now imagine that this is being focused into some volume of brain tissue, we have a small volume that gets excited by our two-photon excitation beam that we focus in here, and we can then measure the emitted photons from that, and the way that we can turn that into an image is then by moving this excitation volume around an X, Y and Z, and we do that through different scanning mirrors, where we can move the beam in the focal plane here. So we move our focal volume around inside the sample, and at the same time, where we know where we're putting that beam, because we drive the scan mirrors, we can then simultaneously collect the emitted photons on a photomultiplier tube, we correlate the position of the X-Y scan mirrors with the amount of fluorescence that's being detected, and then we then move that focal volume, scanning it across the focal plane, in X, Y and if we want we can also change the focal distance, and then we can build up three-dimensional volumes of what we're imaging inside the brain.

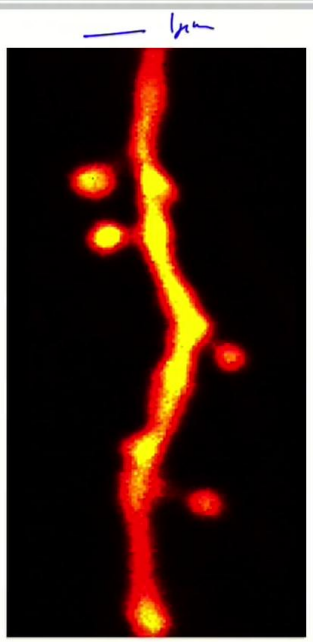
Notes

Summary

19m 12s

In vivo two-photon microscopy





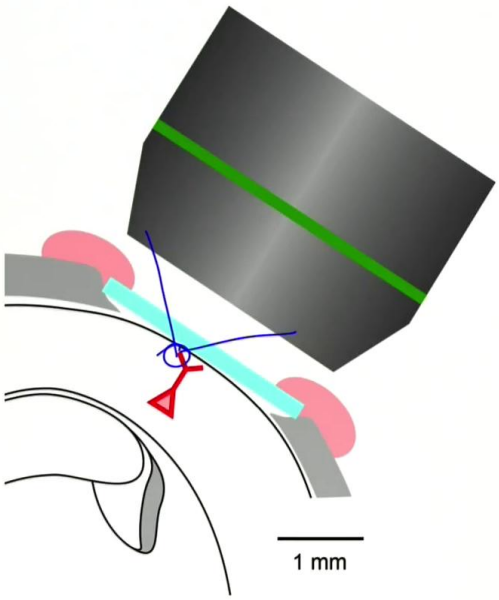
Remarkably, two-photon microscopy works well in the intact living animal. You can replace the bone with glass cover slips, making cranial windows, that can remain stable and clear for months at a time. So you can keep imaging the brain of the mouse over long periods of time, and you can focus your beam down on individual small structures, like dendrites. And here's one example of a dendrite labeled with green fluorescent protein, imaged in the somatosensory cortex of a living mouse. The scale here would be roughly one micron, and here's the dendrite, and sticking out from that dendrite are these dendritic spines that we've already discussed, the sites of where glutamatergic synaptic input arrives on this dendrite. So we can study, at the level of dendrites, spines, the structure of the nervous system in a living animal. And one of the remarkable observations that Karel Svoboda and his collaborators made is that the structure of the brain isn't completely stable. At the fine-scale level of individual spines, it turns out that they come and go, and that also depends upon the experience of the animal. And so on Day Zero, there might, for example be two spines on the dendrite that you're looking at.

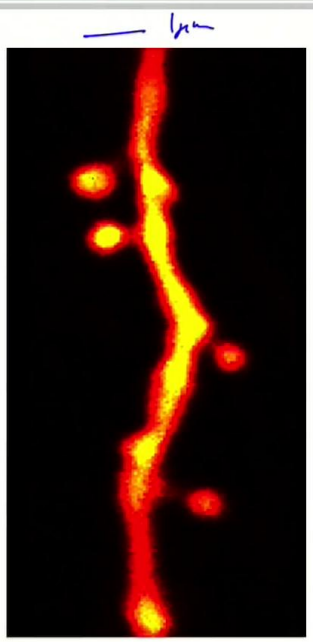
Notes

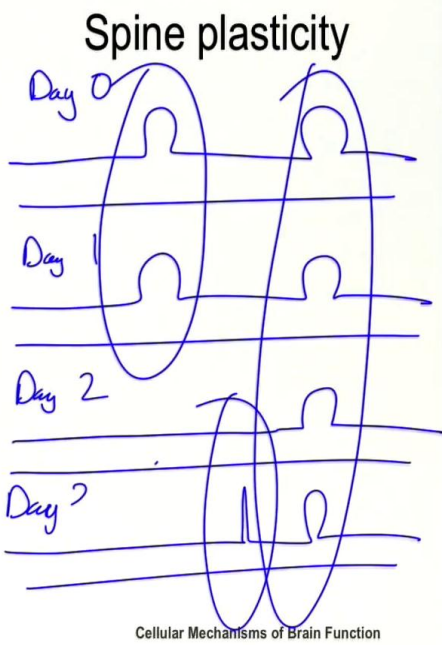
Summary

20m 36s

In vivo two-photon microscopy







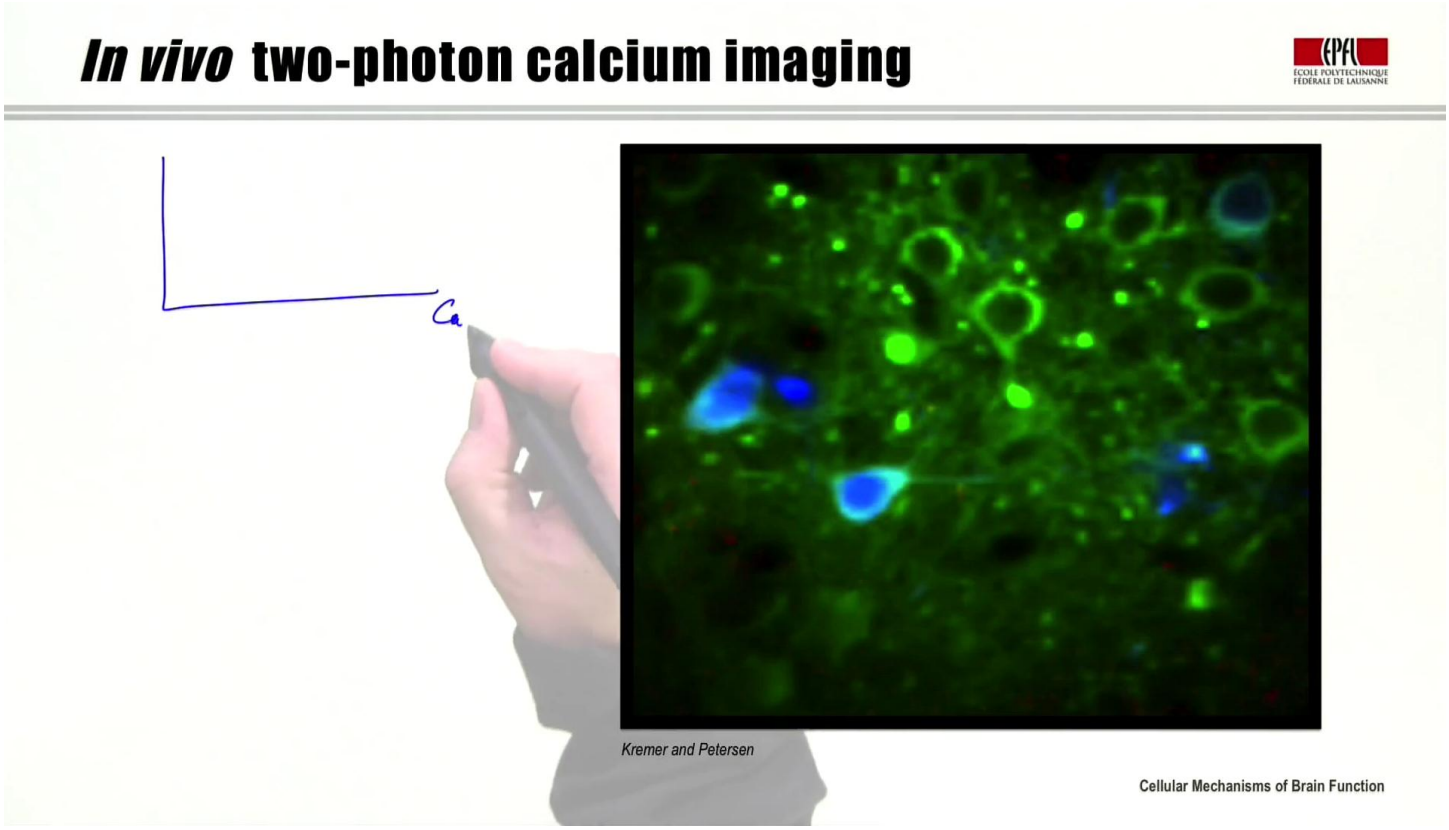
On the next day, they might still be there, indicating some form of stability. Then on another day one of them might disappear, and the other one might stay. And on yet another day, there might be the appearance of a new spine. And so one can then measure the change in spines, and one can then infer that there has been changes in the synaptic wiring diagram of the brain, so that some of the inputs to the cell remain stable for a couple of days, and then disappear. Others will remain stable throughout the lifetime of an animal, and others, new spines will be made during, perhaps interesting learning experiences that the animal has had.

Notes

Summary

22m 01s

In vivo two-photon calcium imaging

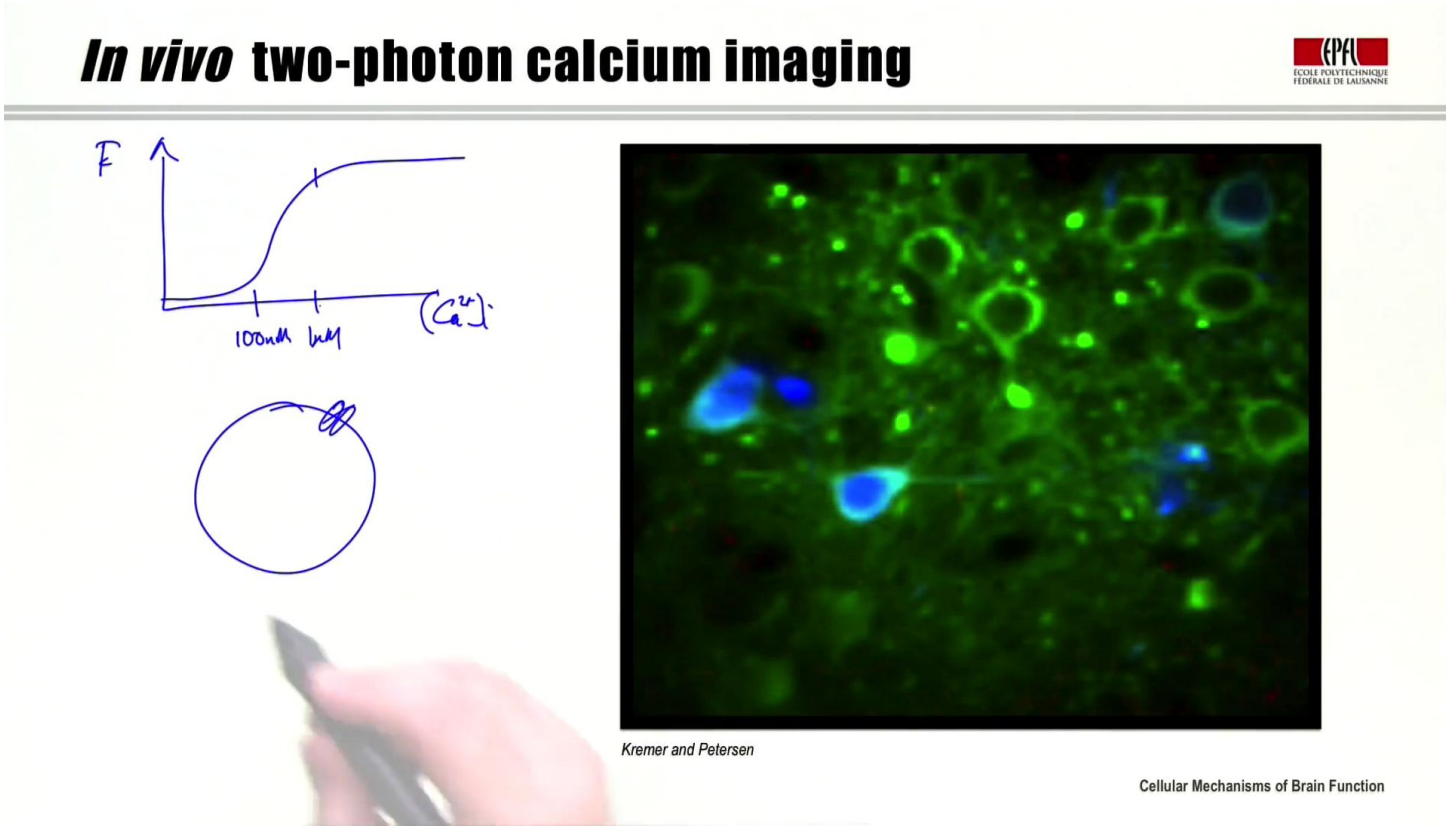


Imaging the structure of the nervous system is, of course, extremely interesting. But even more interesting is to image the neuronal activity of these cells, and synapses at high temporal resolution. And one way in which this can be done is by using calcium imaging. And so calcium turns out to be a relatively good thing to study. It's relatively easy to make calcium-binding molecules that change their fluorescence, and Roger Chin initially developed small-molecule calcium-fluorescent sensors that would change their fluorescence depending on calcium concentration. So if this is the calcium concentration, the cytosolic calcium concentration on the X axis, the fluorescence of an indicator on the Y axis, the fluorescence will change, of these calcium-sensitive dyes, and you can make them, so it's in the useful physiological range, where the dye might be relatively weak, fully fluorescent, at resting calcium levels, at around 100 nanomolar, and then might be strongly fluorescent at one micromolar, which would be a sort of a typical calcium concentration that might be reached during neuronal signaling.

Notes

Summary

22m 50s



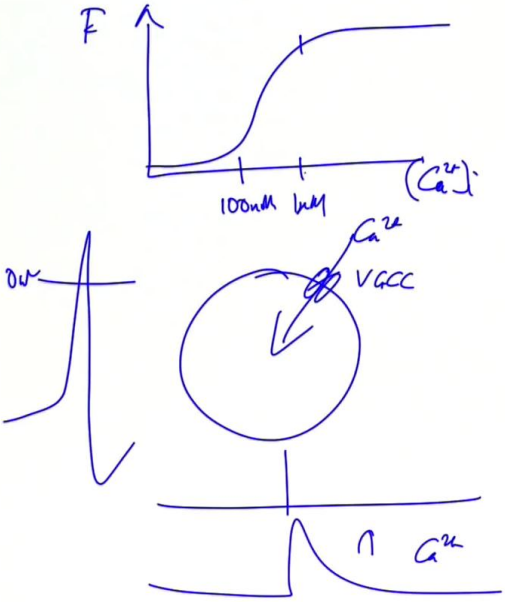
So calcium-sensitive dyes have been made that are both small organic molecules, and Roger Chin also designed the first genetically encoded proteins that were calcium-sensitive, so you can put them inside animals and let those animals live with the calcium-sensitive fluorescent protein, and you can then study the dynamics of these fluorescent calcium indicators over days, looking at how neurons, spines and synapses are behaving. And the interest, of course, in imaging calcium, is that calcium is highly dynamic, and it is a strong regulator of neuronal activity. We already saw that calcium rises are what drives exocitosis in the presynaptic terminal, so neural transmitter release. Postsynaptically, they're responsible for driving synaptic plasticity, and it turns out that for every action potential, there's an increase in the cytosolic calcium concentration that's present both in axons, dendrites and also in the soma. And the reason, of course, is that there are voltage-gated calcium channels present on all the neuronal membranes. And so during the action potential, there's a strong depolarization, beyond -14mV, that activates voltage-gated calcium channels, and during that action potential, calcium then floods into the nerve cell, both in the synaptic axons, but also in the cell bodies.

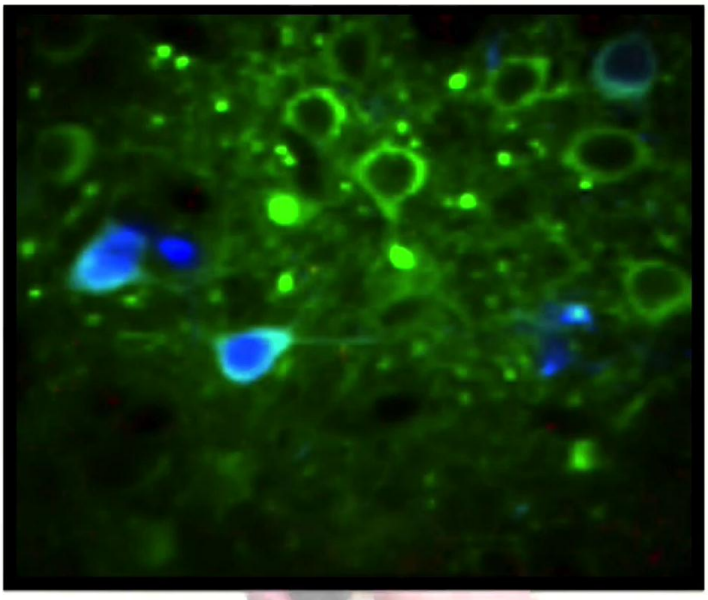
Notes

Summary

24m 05s

In vivo two-photon calcium imaging





Kremer and Petersen

Cellular Mechanisms of Brain Function

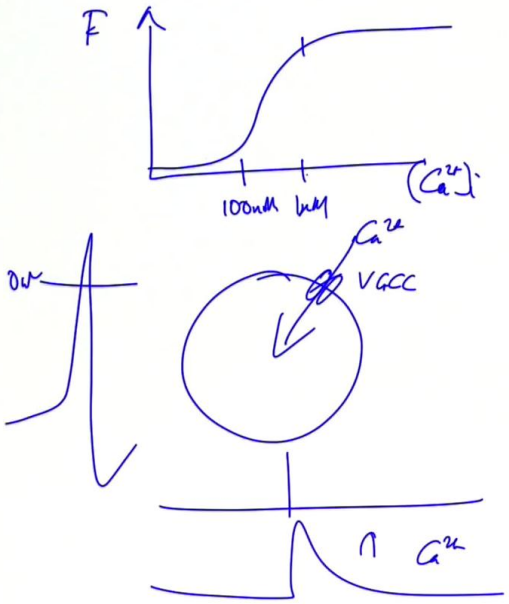
And so whenever there's a spike in a cell, there's also a cytosolic calcium rise in those neurons. And so by imaging calcium you can then infer when cells were firing action potentials, and that's what we're going to look at here. In this movie here, we're looking inside the neocortex, the primary somatosensory neocortex of an awake, behaving mouse. The green that you see here is fluorescence from a genetically encoded calcium indicator that's sitting in the cytosol of neurons. And so here is one neuron, and the cytosol, this ring of green fluorescence, surrounds the nucleus, so the nucleus excludes the fluorescent protein, and it's just the cytosol that's labeled. You'll also see other brightly labeled structures, and these are dendrites that are coming out of the plane of the imaging that we're looking at. So the green fluorescence relates to the calcium-indicator expression. Blue, in this image here, indicates genetically defined populations of gabaergic neurons, and superimposed on this we're going to have an orange color, that indicates changes in the fluorescence of the calcium indicator, that then reveals the function of these cells in real time.

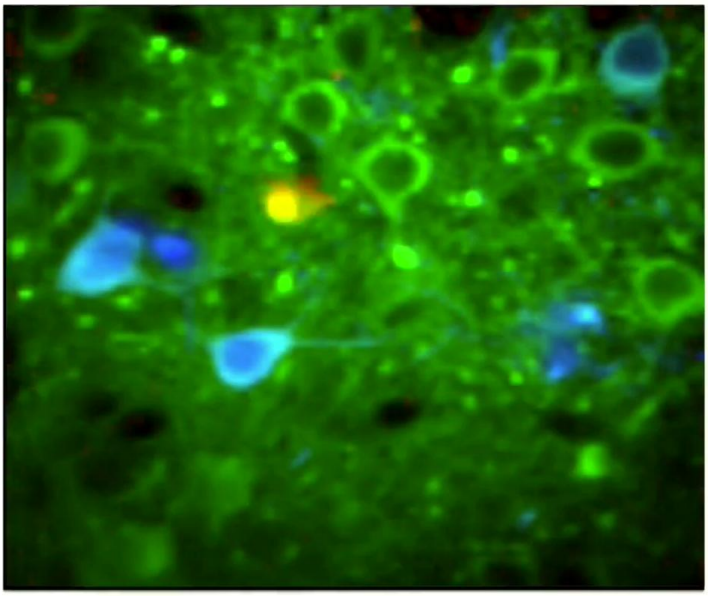
Notes

Summary

25m 36s

In vivo two-photon calcium imaging





Kremer and Petersen

Cellular Mechanisms of Brain Function

So here's the image of the brain, and you'll see that cells are lighting up, flashing, as they fire action potentials. You'll also see that the activity is relatively sparse, and that's typical of the neocortex, that only a few cells are active at any given time. And you'll also see that processes are lighting up, individual little dendrites are lighting up, and also, together with some of the soma, you'll see the dendritic structure of those cells also lighting up. So whenever there's an action potential that back propagates into the dendrites, and you also see dendritic calcium signals associated with action potential firing.

Notes

Summary

26m 57s

Fluorescence imaging of brain function

Fluorescent probes (both chemical and genetic) are being engineered to monitor many different cellular activities, including:

voltage, Ca^{2+}, second messengers, protein-protein interactions, synaptic vesicle release, neurotransmitter receptors, …

Different neuronal compartments can be imaged:

cell bodies, axons, presynaptic boutons, dendrites and spines

New techniques are being developed for improving optical imaging at many different levels, e.g. *super-resolution microscopy*

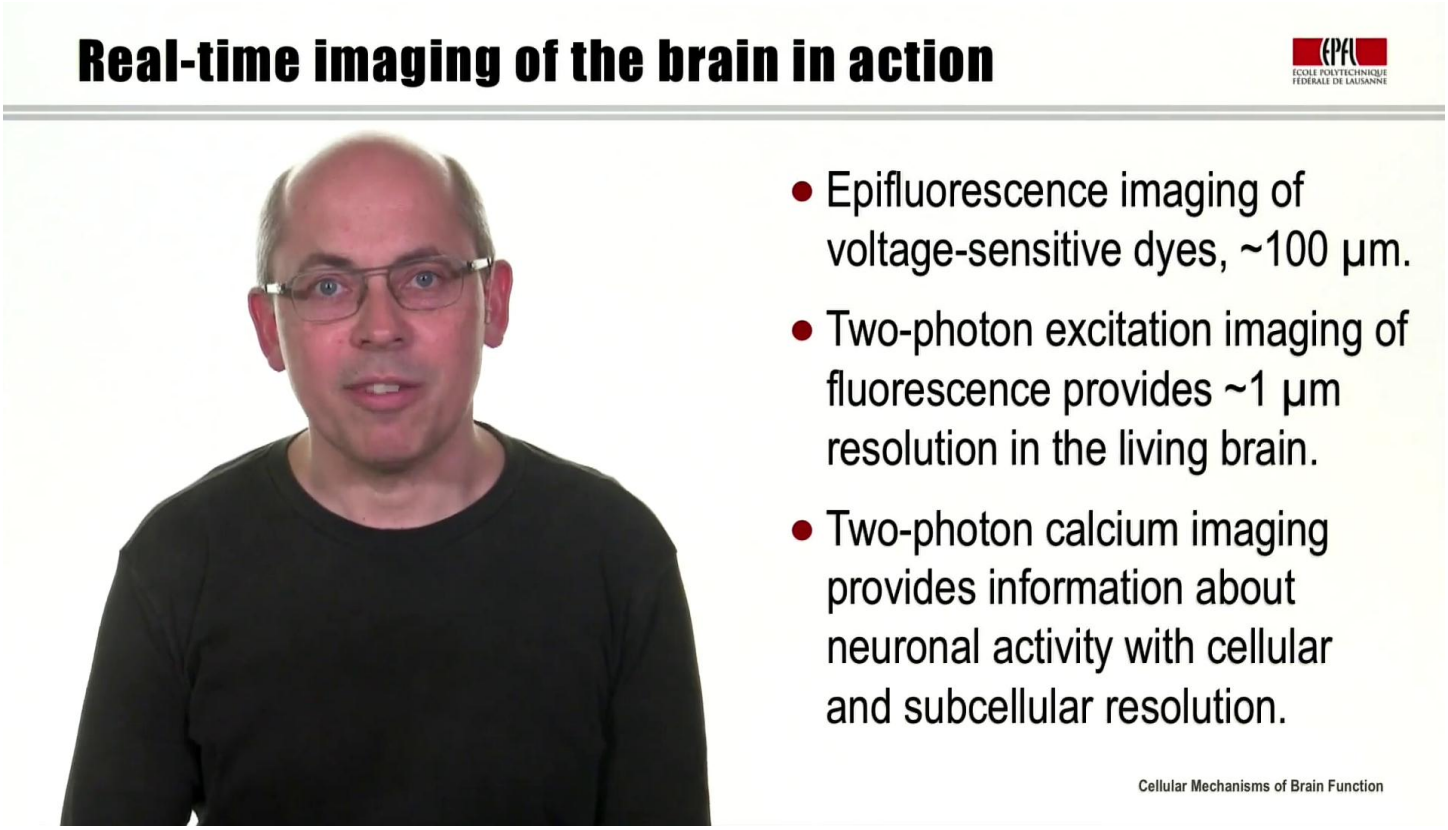
Cellular Mechanisms of Brain Function

Optical Fluorescence Imaging of brain function is extremely powerful, and it turns out that fluorescent probes have been made that are sensitive to a large number of different cellular activities. So we've seen voltage, and calcium as things that drive change in fluorescence in different fluorescent probes, but there are other fluorescent probes that indicate the role of different second messengers, so there are probes that are sensitive to cyclic AMP concentrations that measure different protein-protein interactions, pH sensors that measure synaptic vesicle release, and you can even bind fluorescent proteins onto neurotransmitter receptors, and begin studying the opening and closing of individual ion channels through fluorescent imaging, and that's through both organic and genetically encoded fluorophores. With two-photon microscopy, one can image cell bodies, axons, boutons, dendrites, and spines at high resolution. And newer techniques are even being developed to get even closer to that nanometer scale of resolution. In particular, super resolution in microscopy, or nanoscopy, is an interesting direction for future optical imaging in vivo.

Notes

Summary

27m 33s

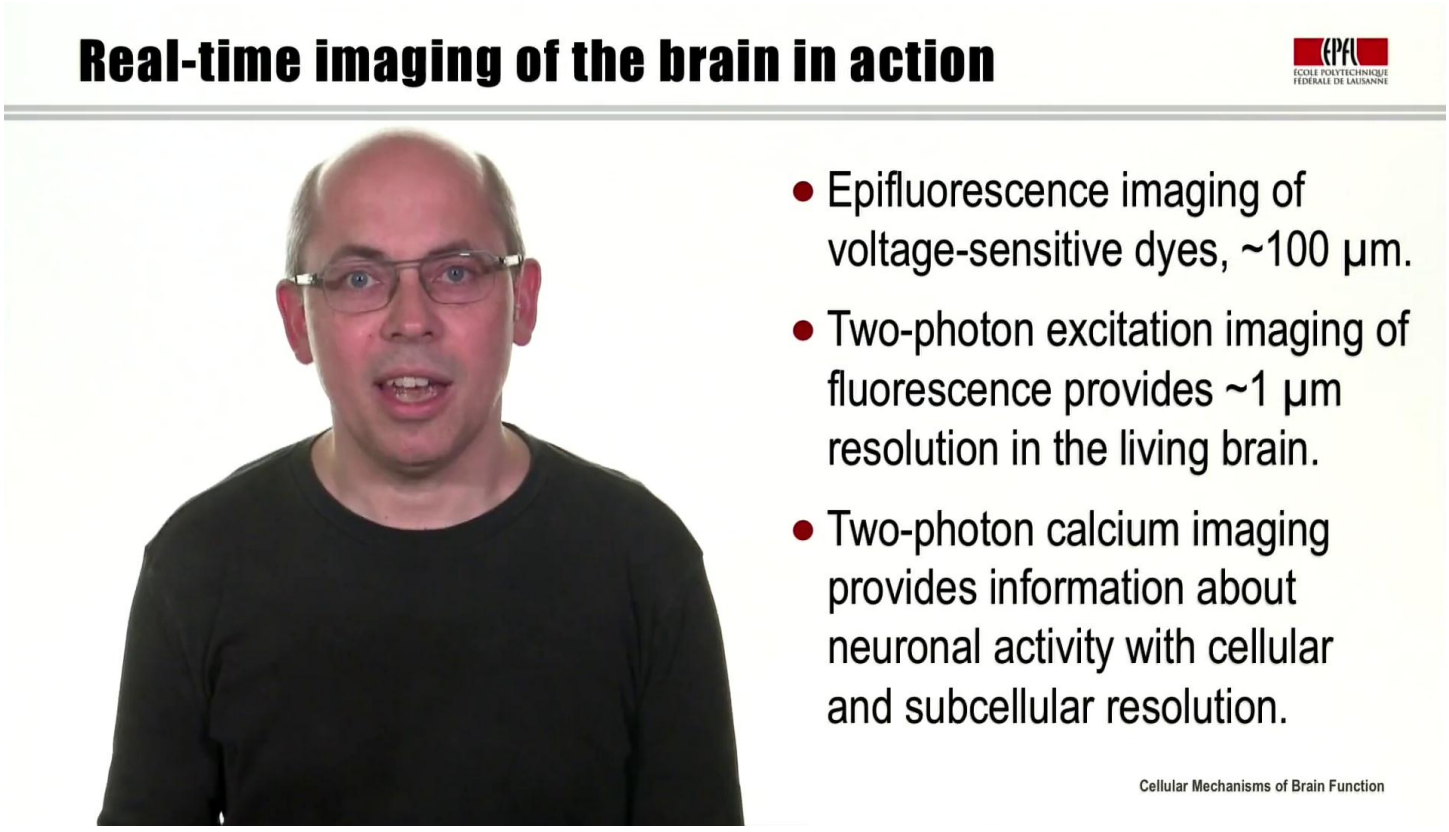


So in this movie, we've seen that there are two interesting approaches to imaging the brain in action. There is a wide-field single-photon epifluorescence approach, where we can image the dorsal surface of the neocortex of the brain, and we can then get something like 100-micron spatial resolution. And if we're looking with voltage-sensitive dyes, then we have millisecond spatial-temporal resolution about the dynamics of activity in the neocortex, that we can then directly compare to the behavior of the mouse. We can also use two-photon microscopy, through these non-linear optical techniques, that give us optical sectioning deep in the brain, get rid of the scattering of the light and the out-of-focus fluorescence, and then we can image with micrometer and submicrometer resolution the structure of the brain. And if we put in different indicator proteins or fluorescent molecules, that change their fluorescence depending upon activity, we can then, for example, measure calcium signals in individual neuronal compartments, fulfilling that dream that we had of measuring neuronal activity with cellular resolution, and also seeing the synaptic activity that links the activity of different neurons in the brain.

Notes

Summary

28m 48s



So in this video we've seen how optical imaging can be extremely useful in terms of defining brain function. It's of course also extremely limited. We can only look at the surface of the brain, perhaps the top millimeter of the brain can be explored through optical imaging, and if we want to look deeper in the brain then we have to become more invasive with endoscopic techniques.

Notes

Summary

30m 06s