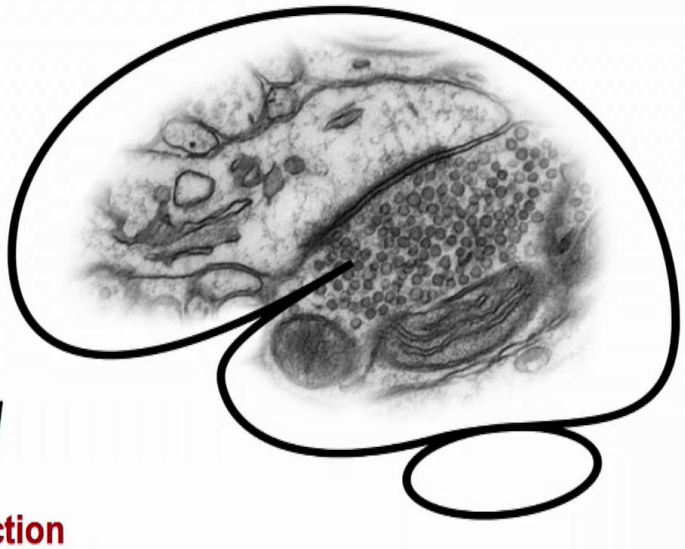
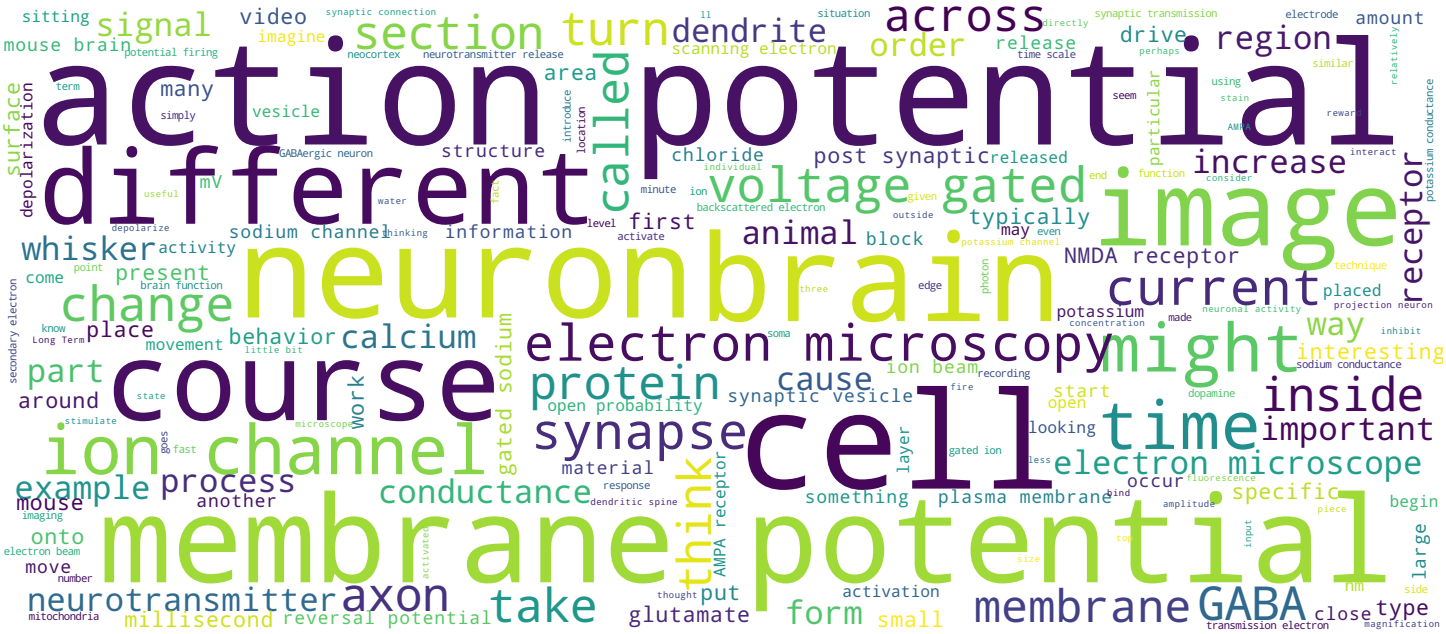


3.5 Electron microscopy

Cellular Mechanisms of Brain Function

Graham Knott



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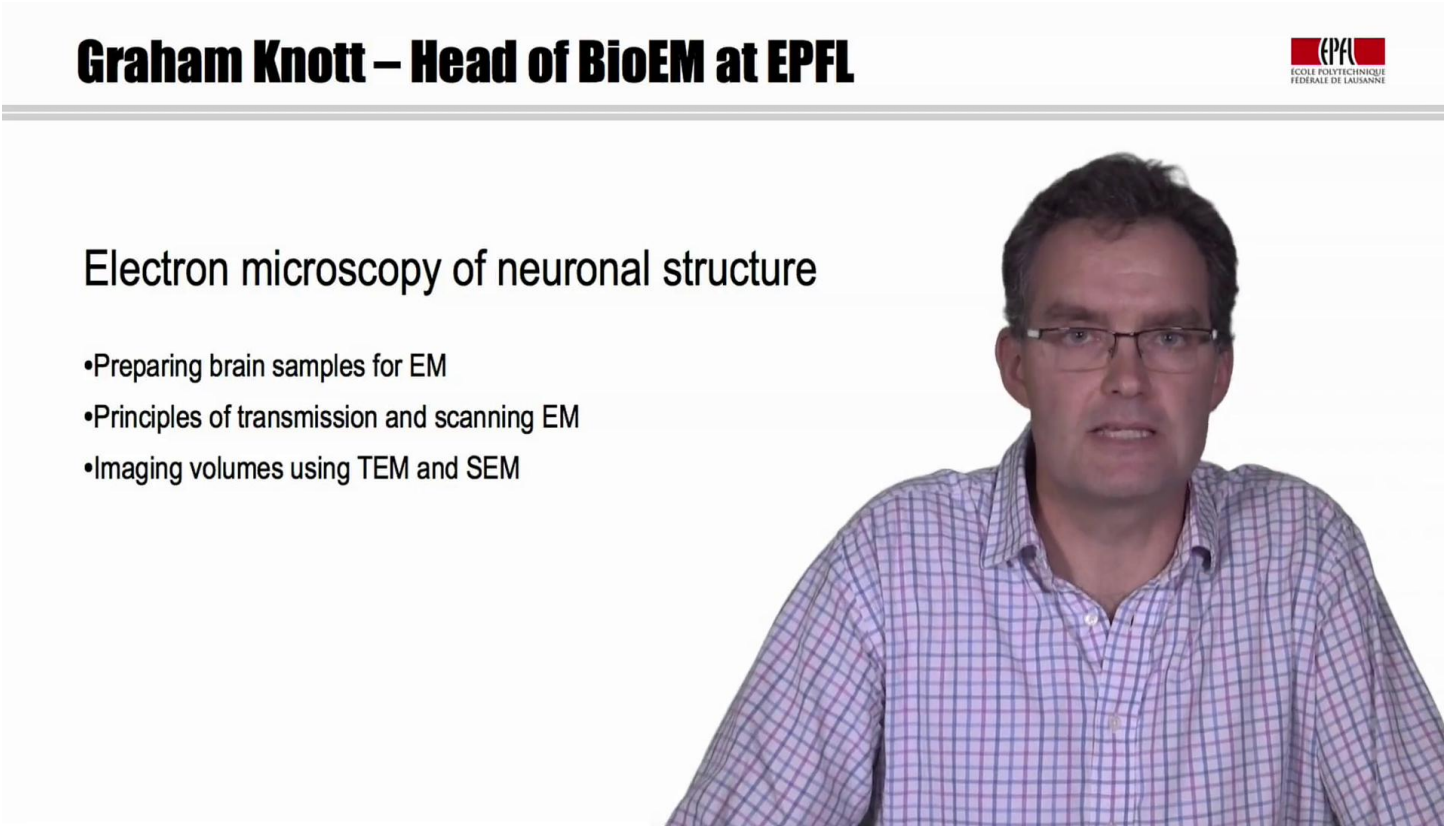
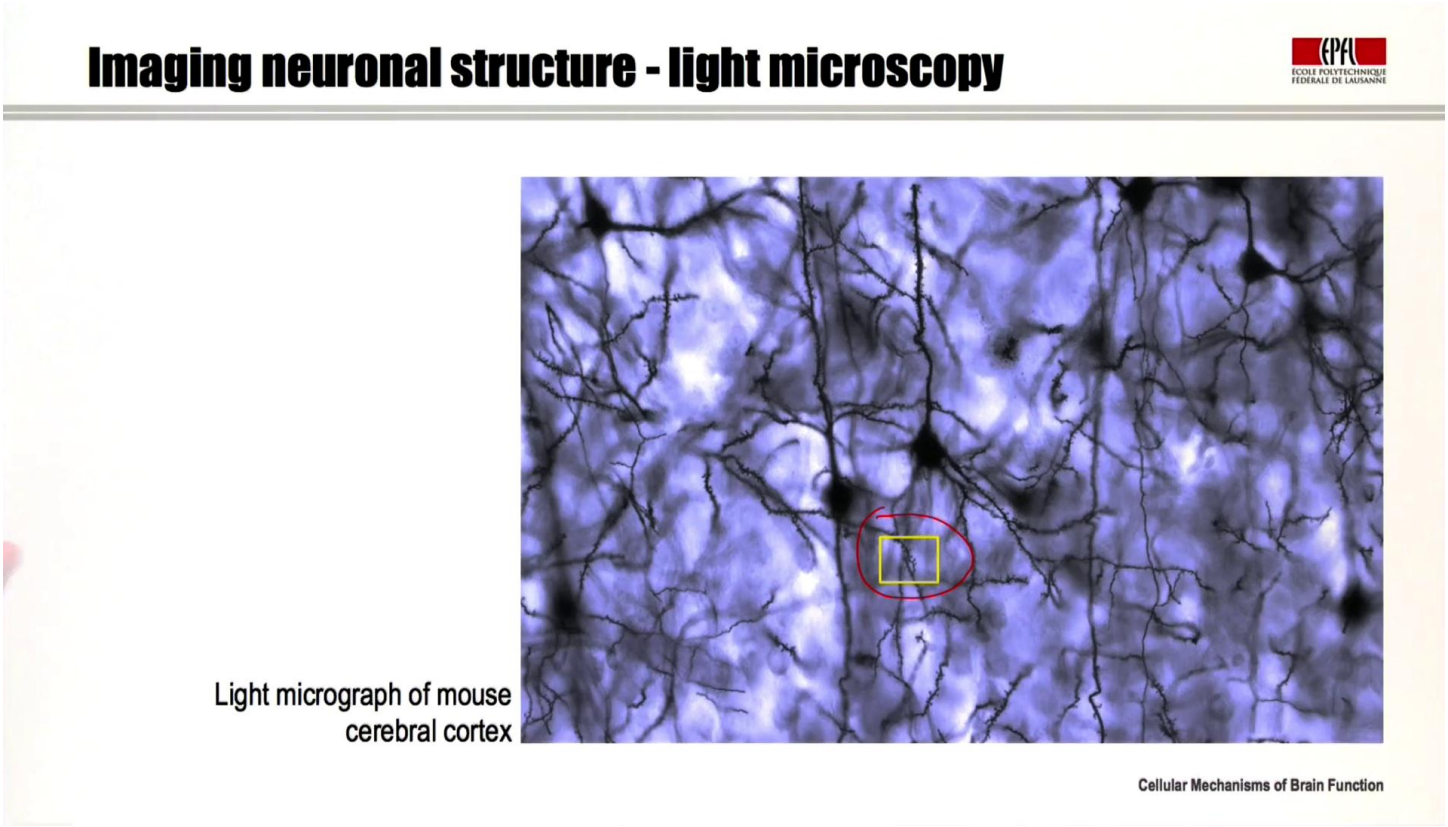


In this video we're going to take a look at the structure of synapses using electron microscopy. And to introduce you to electron microscopy I'd like to welcome Graham Knott, who heads up BioEM at the EPFL. He's a world expert in electron microscopy. Thank you, Carl, and hello, everybody. In this video I'd like to give an introduction to how we use electron microscopy to image biological samples, and in particular I'd like to show you how we prepare tissue from the central nervous system for imaging with transmission electron microscopy, and scanning electron microscopy. So what we will look at is how we prepare brain samples for electron microscopy. We'll look at also the principles of both scanning and transmission electron microscopy, and at the end I will just introduce you to some techniques of imaging volumes of tissue using the current electron microscopy techniques.

Notes

Summary

0m 05s

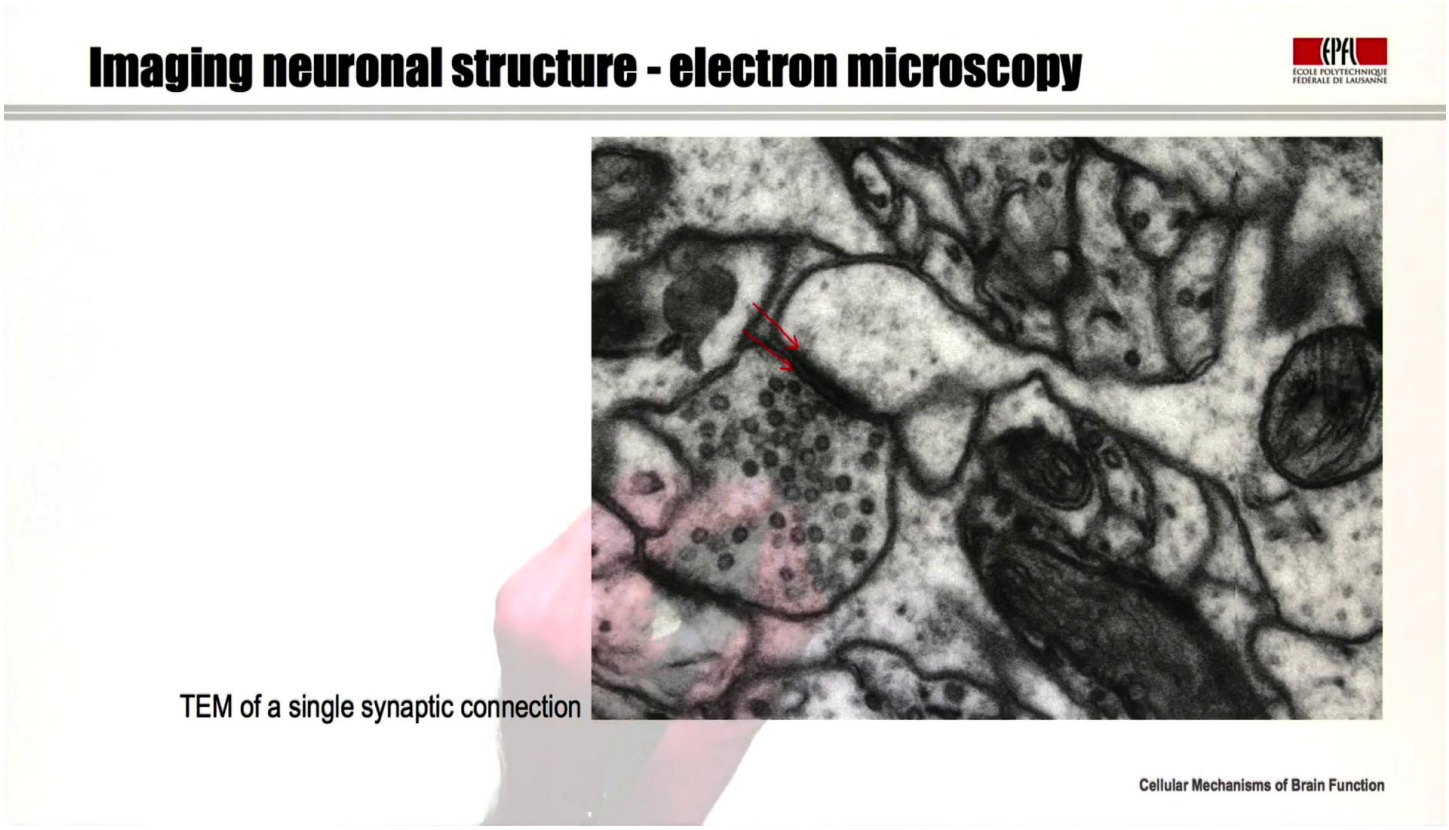


If we start off by looking at a micrograph of the mouse brain, it's a section that has been stained with a silver technique, that reveals just a tiny percentage of some of the neurons contained in this material. We can see neuronal cell bodies, we can also see dendrites protruding from the cell bodies, and we can also see some axons traversing through the material. But if we were to take a tiny piece of this material and image it in the electron microscope, here I show a window of about 15 microns, we would see an image that looks very much like this, showing a jumble of membranes and very heavily stained cellular structures.

Notes

Summary

0m 57s



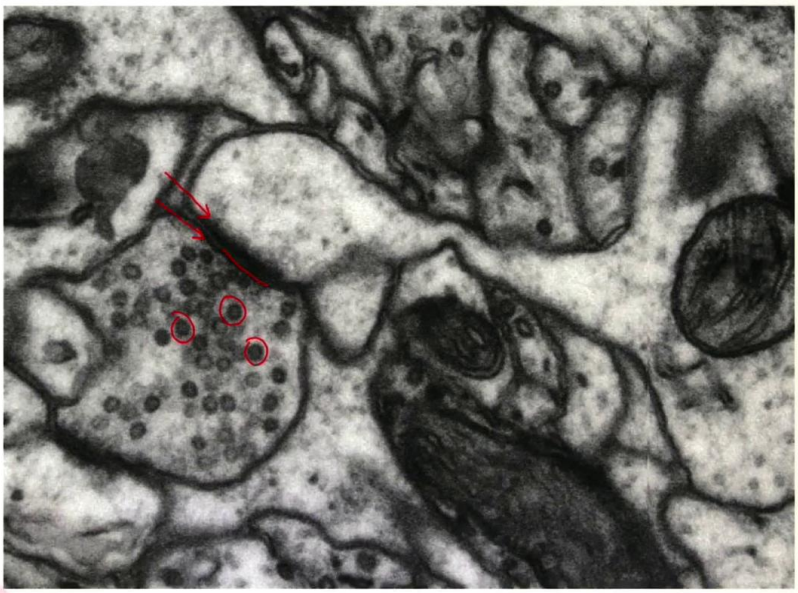
Easily seen in this image are mitochondria, that are seen throughout the material, which essentially shows axons and dendrites that are lying in various orientations. On the right-hand side, we can see a very large dendrite that has been cut almost longitudinally, and in it you can see two mitochondria. You can also make out the thin shapes of the microtubules that run throughout structures like this. If we increase the magnification, and look just at a small portion of the image, for example, this region here, you see on the right-hand side, we have a dendrite that's been cut almost transversally, containing a mitochondria, and inside the mitochondria we can see the cristae membranes. We also have the microtubules in evidence, but protruding from the dendritic shaft we have this long, thin spine neck, and on the end of the spine neck we can see the head of the dendritic spine, and here we find a synaptic connection. This is characterized by a thick postsynaptic density and a thinner presynaptic density, and this is typical of an asymmetric or glutamatergic excitatory connection. We also can see the synaptic vesicles in the presynaptic part, and these are round, and clear.

Notes

Summary

1m 45s

Imaging neuronal structure - electron microscopy



TEM of a single synaptic connection

Cellular Mechanisms of Brain Function

The two synaptic densities are separated by synaptic cleft, and in the presynaptic part we can see that the vesicles are accumulating here around the active zone. So with this technique we're able to see much of the detailed morphology of the brain, such as the synaptic connections, and I would like to explain how we're able to capture images like this from such fragile living material.

Notes

Summary

3m 25s

Considerations for EM of biological structures

- Samples contain atoms of low molecular weight
- Most biological samples contain water
- High vacuum
- Intense heat of the e beam
- Size of Specimen

Cellular Mechanisms of Brain Function

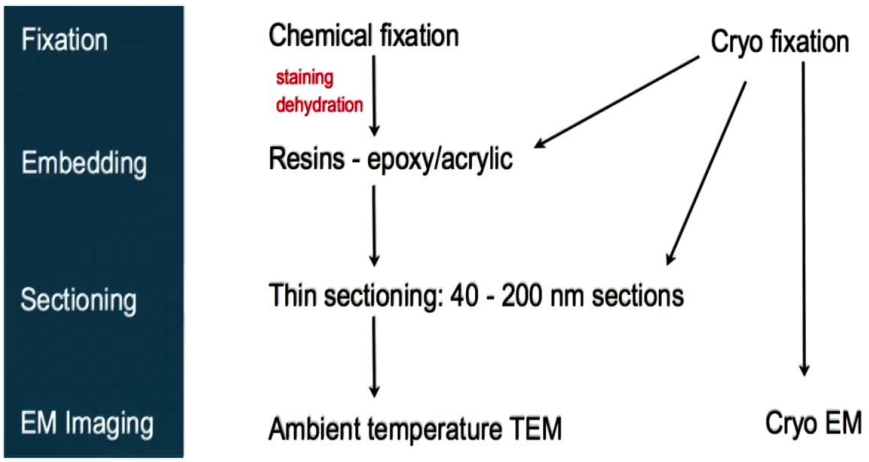
For biological structures, we need to remember, of course, that they're mostly made up of atoms, with a varying low molecular weight. Carbon, hydrogen, oxygen and nitrogen, and there's very, very few heavy atoms. And it's these heavy atoms that interact mostly with the electron beam, so we need to think about staining our structures. Of course most biological samples contain water, and we're going to be putting the sample into a high vacuum, so we need to think about removing that water, or at least stabilizing it. We also need to consider the intense heat of the electron beam, and also the size of the specimen. We have to make sure we have a very, very small specimen that's put into the microscope.

Notes

Summary

3m 54s

Preparing biological samples for EM



Cellular Mechanisms of Brain Function

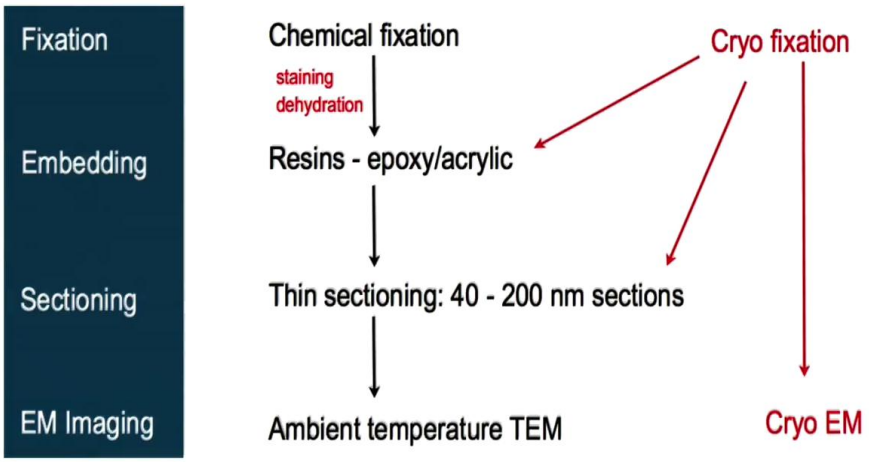
So the general scheme for preparing biological samples is first of all, we need to think about the fixation: we need to stabilize the structure. We may need to think about embedding it in a medium in which it remains stable under the intense heat of the beam. We should consider sectioning the sample, and also we need to think about the EM Imaging technique, which we'll of course going to be using. So, say, for imaging the brain with electron microscopy we generally use a scheme that's outlined here. We generally use a chemical fixation, and after chemical fixation we then use a staining technique, dehydration, and then we embed them in resins, these can be epoxy or acrylic resins, these can then be sectioned very thinly: 40 to 200 nm-thick sections, typically in a transmission electron microscope, and then these are imaged at ambient temperature in the TEM. Of course there are cryofixation techniques that we can use, and we stabilize the structure, obviously using very low temperatures. Then these frozen samples can be embedded in resin with a substitution technique, or we can, in fact, thin section the frozen material directly, or if we're actually able to fix a very, very thin sample, for example, a layer of water containing very small particles, we can directly image a cryofixed sample in the Cryo-TEM.

Notes

Summary

4m 31s

Preparing biological samples for EM



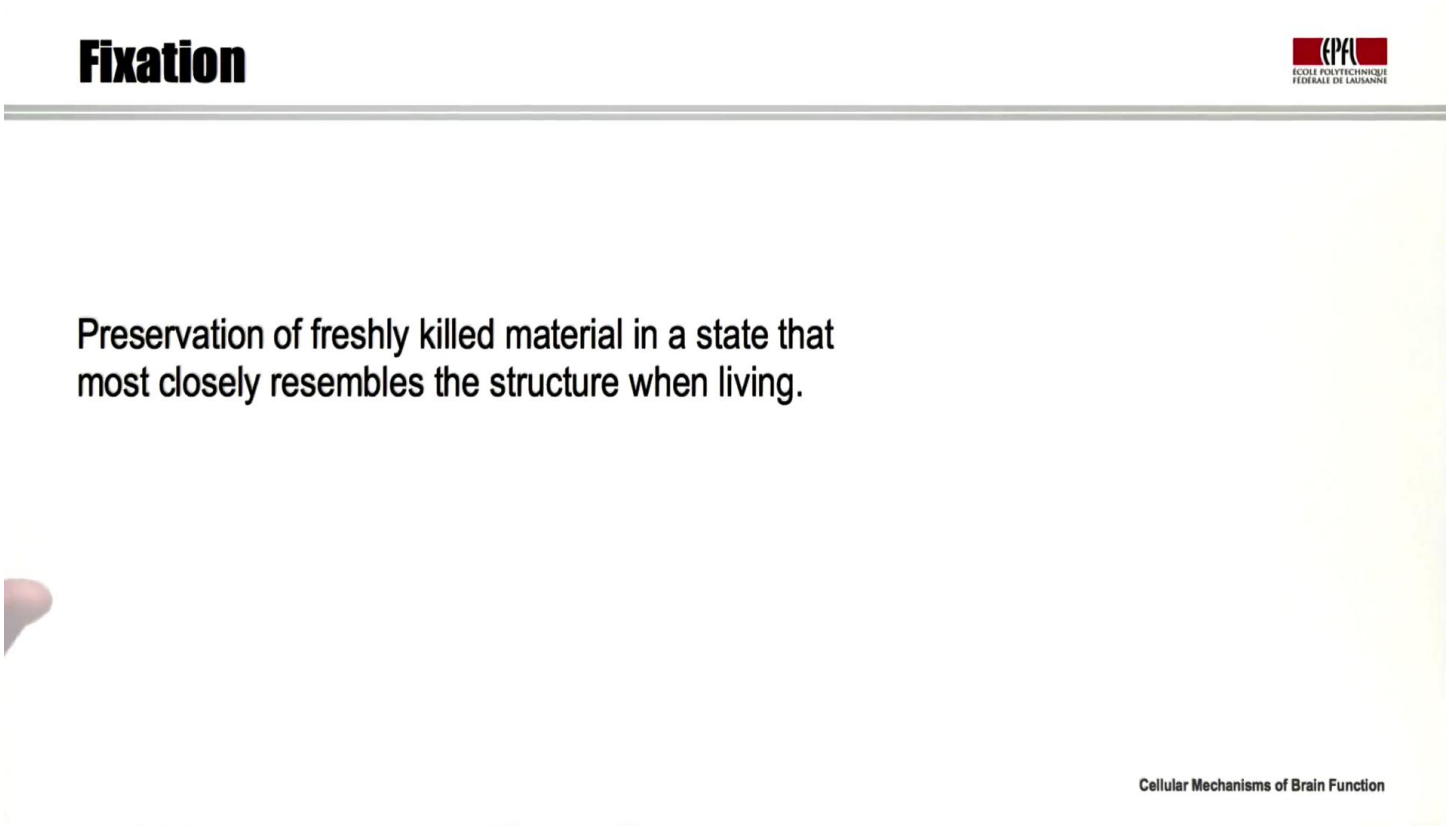
Cellular Mechanisms of Brain Function

However, the cryofixation and cryo imaging techniques are not something I'll be discussing in this video.

Notes

Summary

5m 48s



So what about fixation? Well fixation is the preservation of freshly killed material in a state that most closely resembles the structure when living, and for many thousands of years we've understood that we can use different chemicals to preserve biological structure.

Notes

Summary

5m 54s

Chemical fixation

•Coagulative fixation - acids, ethanol

Typically, in the past, things like acids and alcohols have been used to preserve foods, for example, for many, many months, and even years. But this is a coagulation approach, where proteins coagulate together, and render them very immobile, very inert, and they can be preserved for a long period of time. This is very useful for light microscopy, but not very useful for electron microscopy, where we need to see ultrastructure.

Notes

Summary

6m 09s

Fixation

Chemical fixation

- Coagulative fixation - acids, ethanol
- Non coagulative (polymerizing) - aldehydes

Cellular Mechanisms of Brain Function

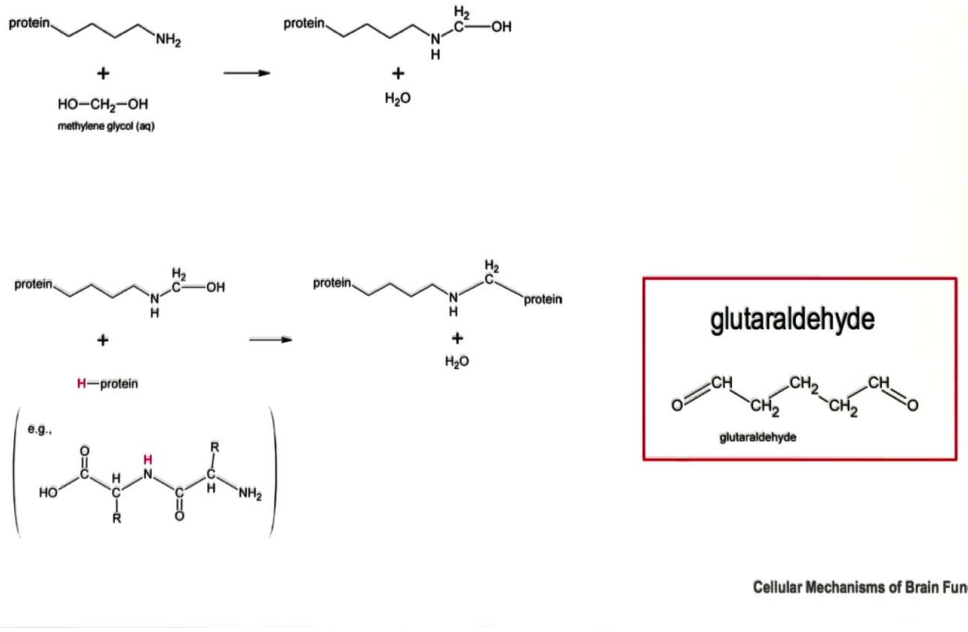
Seeing the ultrastructure we really need to use a non-coagulative fixation approach, and typically we use aldehydes. Now the aldehydes that we use are mostly things like formaldehyde, and for electron microscopy, we also use a lot of glutaraldehyde. And of course one of the considerations is that we need to introduce the fixative very rapidly into the system. So on the right-hand side we see a fixed human brain, and this has been fixed quite rapidly by replacing the blood with fixative. This clears out all the blood, and it provides fixative into the tissue directly to the tissue, very, very quickly, via the capillary beds.

Notes

Summary

6m 35s

Mechanism of action of aldehydes



Cellular Mechanisms of Brain Function

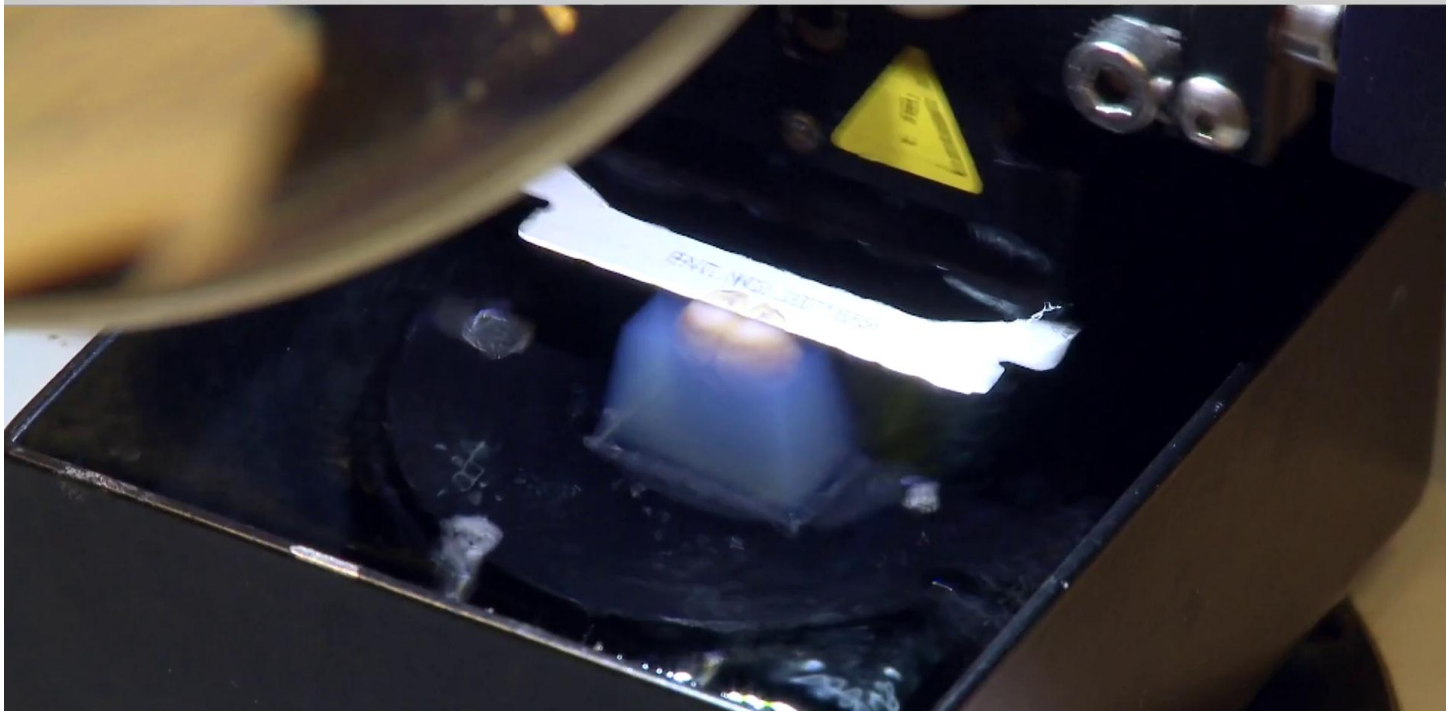
So let's have a look at how aldehydes fix biological material, and essentially they polymerize proteins. Here we have the paraformaldehyde molecule, which in water forms this methylene glycol compound. This then interacts with the amine groups that are found on proteins to form, essentially, a methylene bridge, and it's this part of the protein molecule that can then interact with other proteins, bind them together, and form very, very long chains of proteins, and to polymerize proteins into huge complexes that can then trap lipids, carbohydrates, and nucleic acids. This essentially binds them up and renders them immobile. In electron microscopy, we use also the glutaraldehyde fixative, and glutaraldehyde has these aldehyde groups, one at each end, linked with this chain of methylene bridges. And here we have the opportunity to form much more cross-links, and therefore a much stronger fixation.

Notes

Summary

7m 14s

Staining and embedding brain tissue for EM



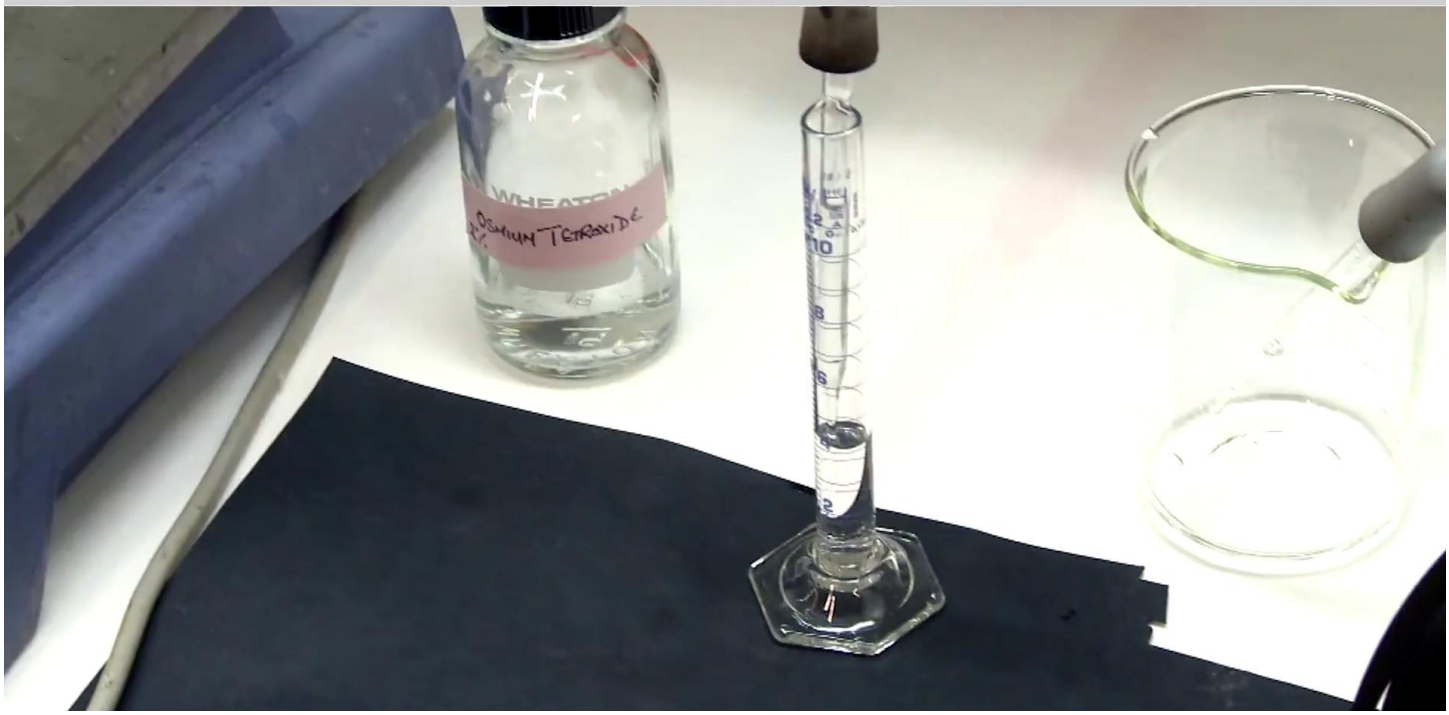
So now I'd like to show you how we prepare a piece of brain tissue for looking with the electron microscope. And here we have a mouse brain that's been perfusion-fixed with paraformaldehyde and glutaraldehyde. It's sitting in a petri dish with PBS. So we take the brain, and we just dry it a little bit, take off the excess buffer. We then push it into a small holder containing molten agarose, and this agarose allows us then to just position it at the right angle in the mold. This agarose is then solidified on ice, and after a few minutes we can take it out of the mold and then cut away the excess agarose so we can get better access to the brain. And once this block is of an ideal size it can then be stuck with superglue to a metal holder. The metal holder is then placed into the vibratome, so it's then put into the bath of the vibratome that's containing a buffer, and this machine simply contains a vibrating razor blade that moves across the surface of the block, cutting sections of the brain. And this can cut sections of between 50 and a few hundred microns thick, and it allows us to get access to exactly the brain region we want, with these very thin slices that we can then image directly under the light microscope to actually check whether we're in the right region of the brain.

Notes

Summary

8m 22s

Staining and embedding brain tissue for EM



But if we'd imaged it with a fluorescent microscope we could also see fluorescent markers. So these are placed into wells, these plastic wells, which we can image very easily under a dissecting microscope, and we can select the sections that we want. The process now is to first of all wash the sections in buffers like cacodylate buffer, which we use a lot for electron microscopy, which are very nonreactive to some of the later contrasting agents that we use. And of course all this work is done in a fume hood. So at this point we need to stain the material, so we introduce heavy metal stains into the fixed material, and typically, for electron microscopy, we use osmium tetroxide, which will stain very heavily the membranes and the proteins. We use uranyl acetate, which typically is a very basic stain which will reveal the DNA and RNA, and we also use compounds like lead citrate, which will stain very heavily glycoproteins like starch and glycogen. So again, these are very toxic compounds, and we need to work in a fume hood. The first process is typically to introduce osmium tetroxide, then we have a rinse of uranyl acetate, and then we may use some lead staining, but often the lead staining's done afterwards.

Notes

Summary

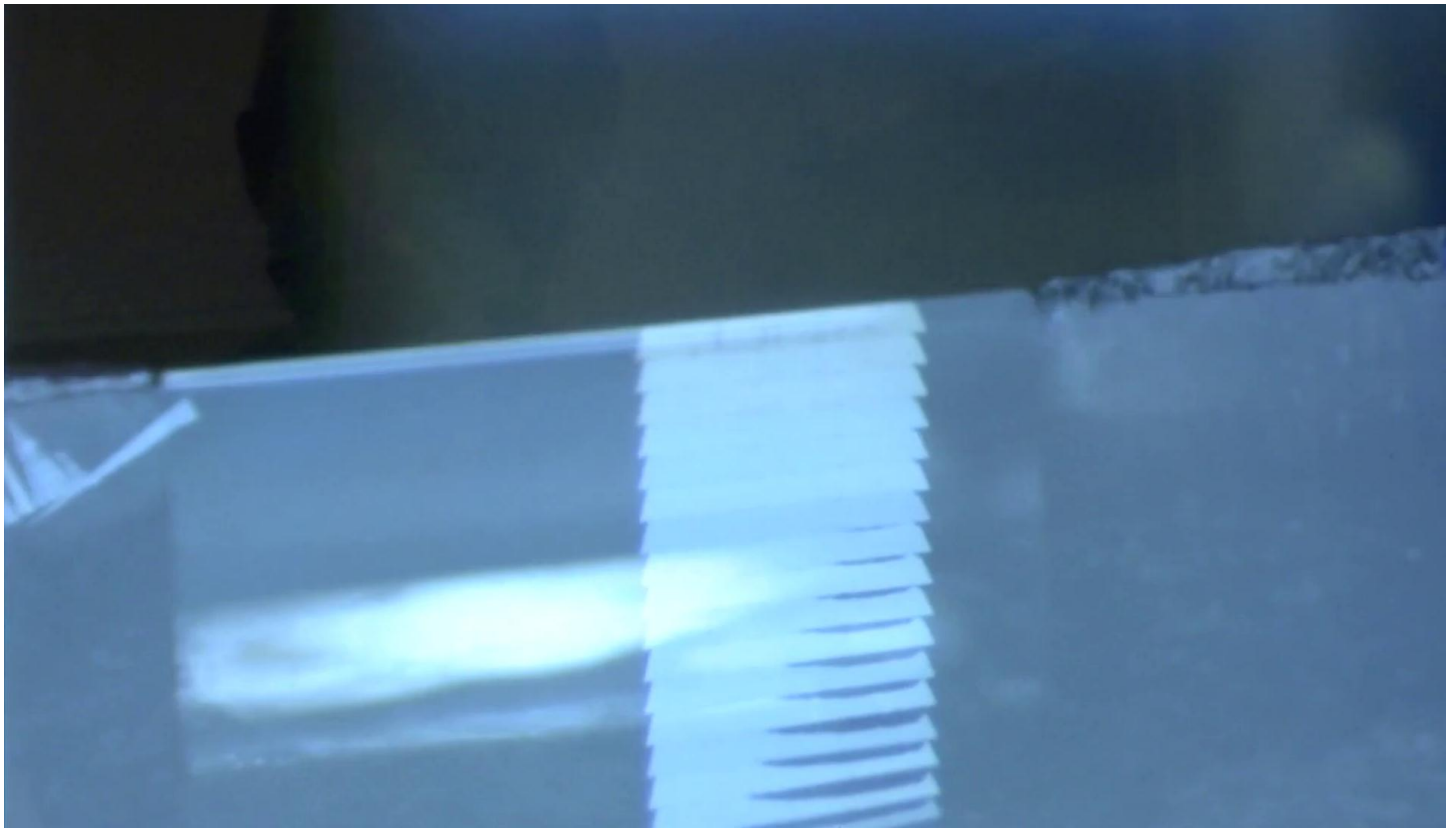
10m 03s

And introducing these heavy metals gives us these very, very densely stained sections, so they typically become black, very, very opaque. There's then a process of removing the water, so we dehydrate the section through a series of increasing concentrations of ethanol, and then once we're in 100% ethanol, we can then start to introduce a resin. And here we see we're using an epoxy resin, and this epoxy resin is mixed with the 100% alcohol, and over a process of many hours the resin infiltrates the tissue completely, there's a removal of any of the ethanol, and this pure resin-embedded sample is then placed onto microscope slides which have a mold-releasing agent dried onto the surface. These are then flattened with a microscope slide on top, and this "sandwich," containing the sections in between, in the resin, is then placed into an oven, and the resin is polymerized at 60 ℃ . This polymerization process takes about 24 hours to fully harden. What we end up with is a section of plastic, essentially, in which we've embedded our brain sections. We can then place this under a dissecting microscope, and we're very easily able to see the regions of the brain that we're interested in.

Notes

Summary

11m 27s



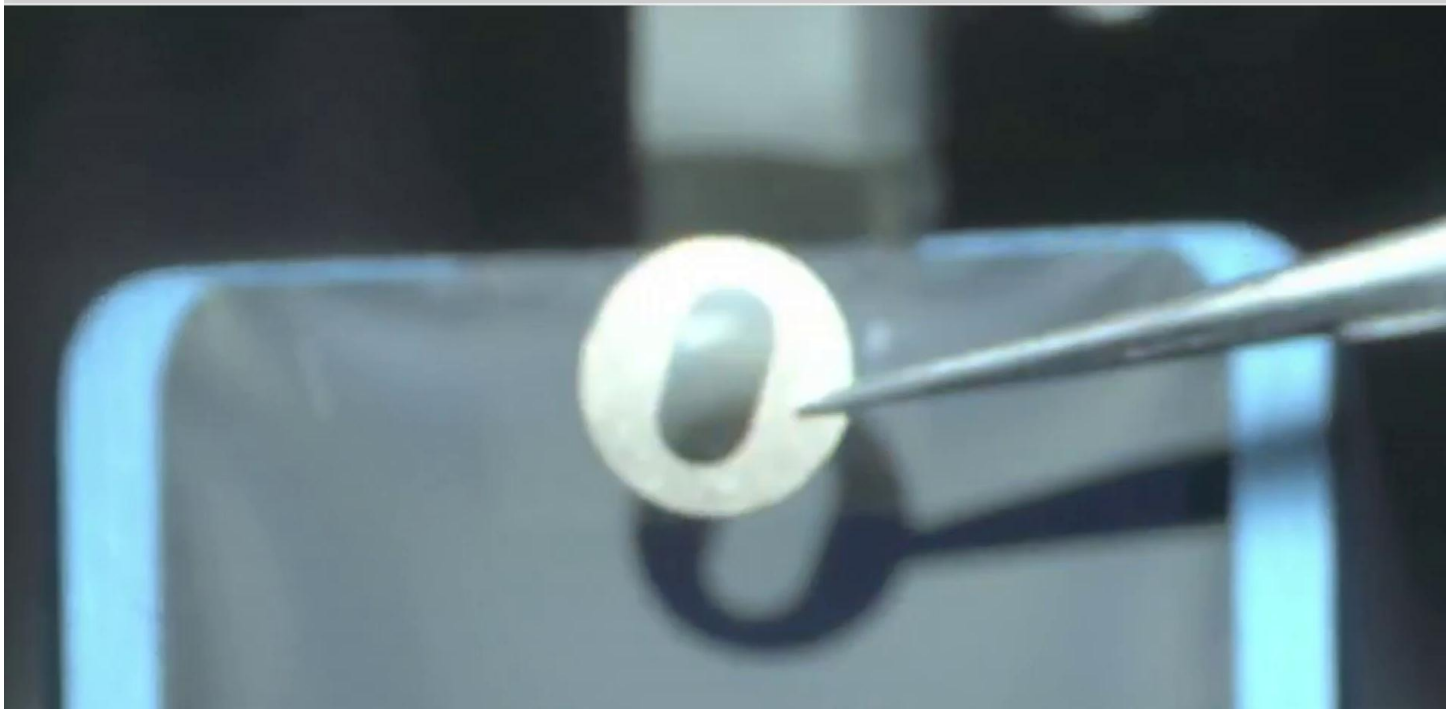
We can then scratch the surface, make a mark of the region that we'd like to image with electron microscopy, and then just taking a razor blade, we can cut away this region from the rest of the brain section, and this piece, this very tiny piece of brain, can then be stuck onto a blank resin block, again with superglue. This blank resin block is then ready for trimming and cutting in the ultramicrotome. So the first approach is to very firmly hold it in a sample holder of the ultramicrotome, and then place it underneath the dissecting microscope, and then, again with a razor blade, just trim away any of the excess material that we don't require. Now this can be a long process, to very precisely make a block of the exact region needed. This is then placed in the arm of the ultramicrotome, a diamond knife is then put in place, and this diamond knife has a very sharp 45°-angle blade, as you see marked on the side of this blue holder, and the block then passes in front of this blade, moving forward at a few nanometers each time. And here we can see 40 nm-thick serial sections being cut by the diamond knife, and floating onto the water that's contained in the bath of the diamond knife.

Notes

Summary

12m 56s

Cutting thin sections for transmission electron microscopy



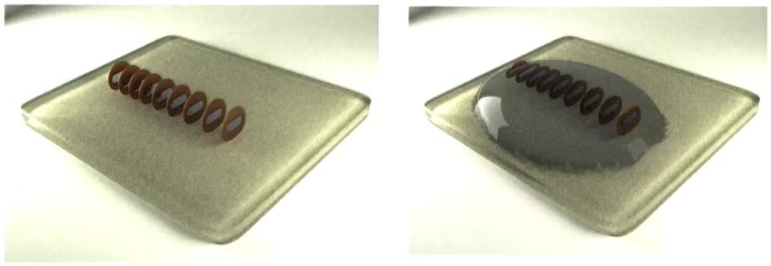
We can then take these manipulators, which are simply very fine hairs stuck to wooden sticks, and these are able to manipulate the sections, and push the sections towards the microscope grid, or the slot grid, onto which the sections will stick. Now these grids, or these slot grids, are simply an aperture across which there's a very thin membrane of polymer, and the sections will stick onto this aperture. You can just about see the sections here, these gold-colored sections, sitting across that hole.

Notes

Summary

14m 33s

Post staining of ultrathin sections



Grids held in plastic holders and flooded with stains
(uranyl acetate and lead citrate)

Cellular Mechanisms of Brain Function

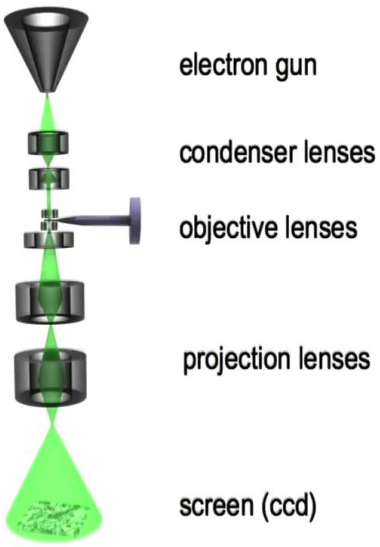
This is then dried for a few minutes, and then this is now ready either for further staining, or for imaging in the electron microscope, so once the sections have dried, we can also stain them again, so post-staining of ultrathin sections, where they're held in a plastic holder, and they can be then flooded with different stains, for example uranyl acetate and lead citrate, to again stain structures like the RNA, the DNA, and also glycoproteins.

Notes

Summary

15m 05s

Transmission electron microscope



Cellular Mechanisms of Brain Function

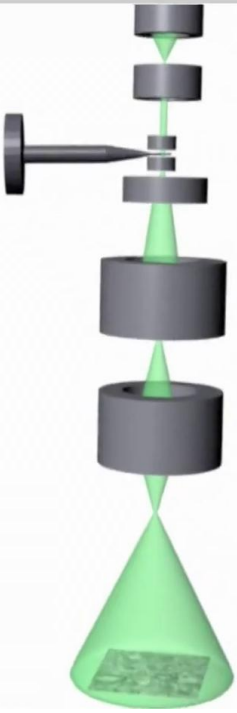
So we can consider the transmission electron microscope as being very similar to the light microscope, and instead of photons being produced we have electrons, which pass through a condenser-lens system. They then pass through the objective lens, and then through a projection system which will then magnify the image onto a screen or the CCD camera.

Notes

Summary

15m 35s

Transmission electron microscope



We can have a very quick look at a movie of how-- of putting the sample into the electron microscope. The sample goes into the objective-lens system here, the beam is then switched on, and an image is then projected down onto the screen at the bottom.

Notes

Summary

15m 56s

Transmission electron microscope

And we can see here that the heavily stained regions, so the mitochondria, the membranes, are appearing black, and the regions that are less stained, the cytoplasm, for example, is much lighter.

Notes

Summary

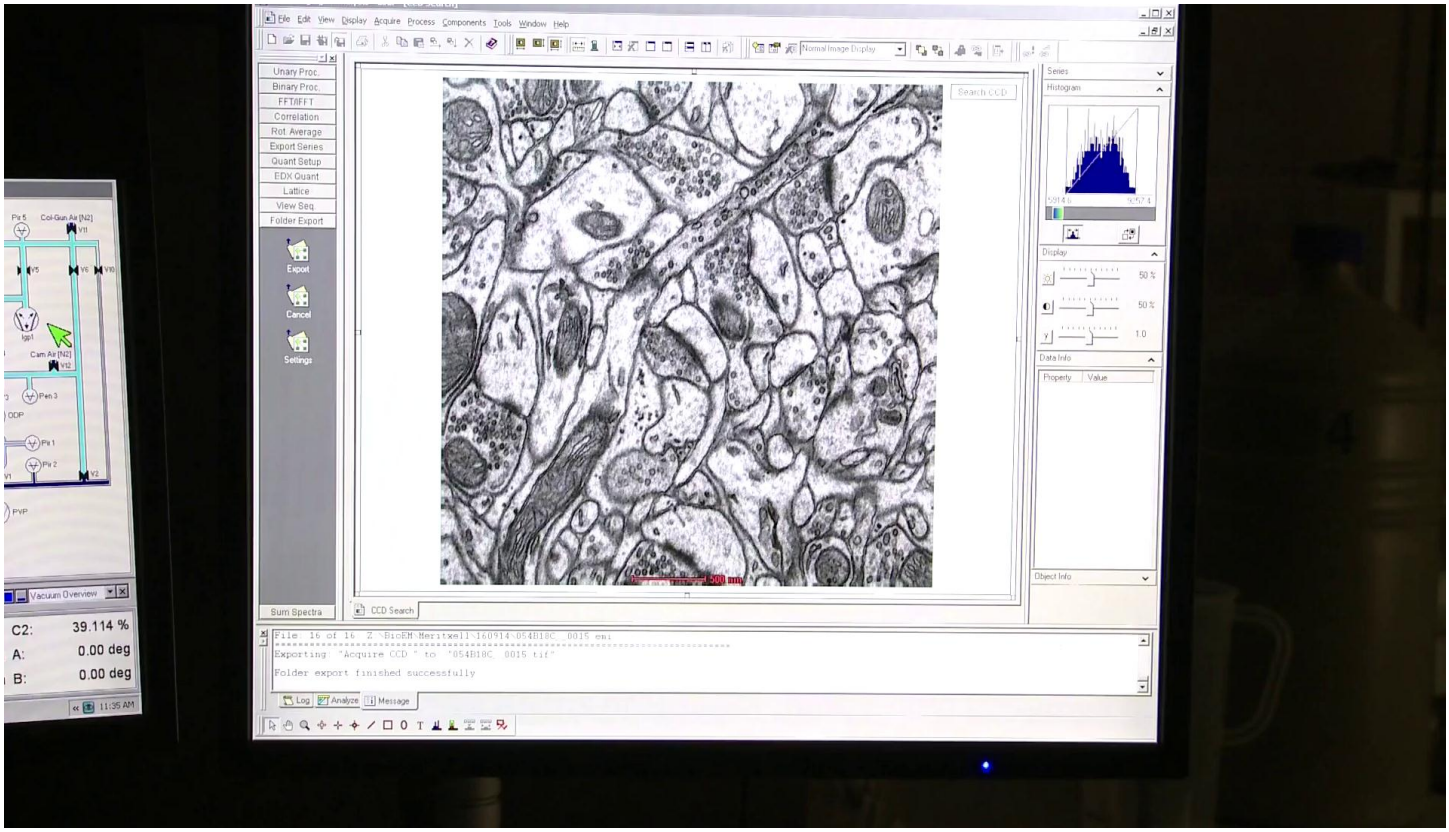
16m 18s

What we can do is just have a quick look at how we do this practically in the lab, and look at a movie of Stephanie putting a sample into the electron microscope. First of all, we place the very small grid onto the sample holder. The grid is then clamped in place so that it doesn't, obviously, move in the microscope. This holder is put in at the level of the objective lenses. It's then put in place, and then into a primary position, where air is pumped out, and then where that air is pumped out, then it's placed right into the column of the microscope. As I said, we have a microscope where at the top we have the cannon producing the electrons, we have a condenser-lens system, then we have the objective lenses where the sample goes, we have the projection lenses, and then, of course, we have a screen. And we can see this phosphorescent screen is being illuminated by the electrons falling on it, and we can move this, the grid, around in the stage and see our sections, you can see the edge of our sections here, and if we turn up the magnification we can already start to see a fairly high magnification, the structure that we have in our thin sections.

Notes

Summary

16m 31s



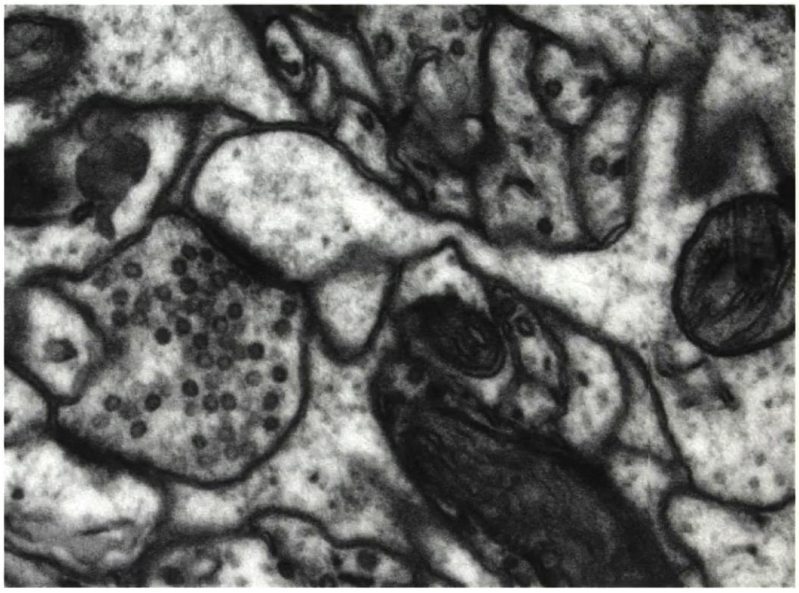
Of course now with CCD cameras attached we can look directly on a computer screen at the samples in the microscope. And here we can see at a low magnification some brain tissue. As we turn up the magnification we can start to see much, much more detail in our section, and here in the middle we can have a synaptic contact, an asymmetric synaptic contact from our piece of mouse brain.

Notes

Summary

17m 48s

Imaging neuronal structure - electron microscopy



TEM of a single synaptic connection

Cellular Mechanisms of Brain Function

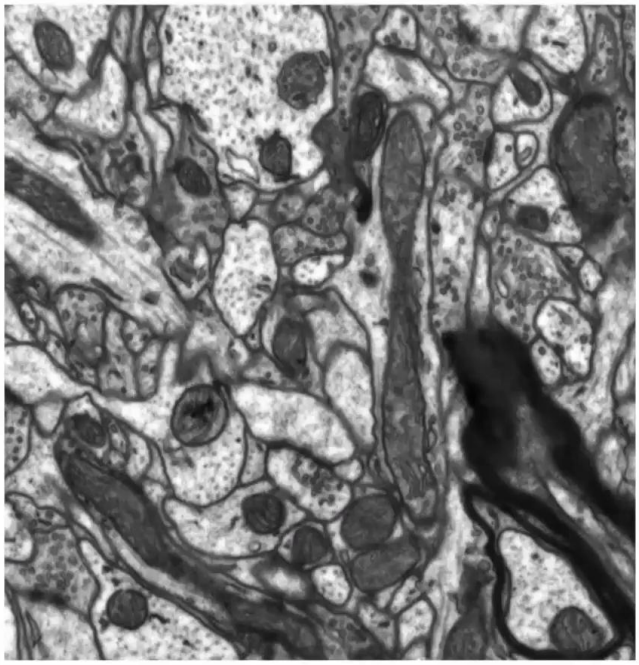
So with this technique of transmission electron microscopy we can generate images, like the one we see here of a section, through this asymmetric synapse on the spine head. And we can get a lot of information from just this two-dimensional image.

Notes

Summary

18m 17s

Imaging neuronal structure - electron microscopy



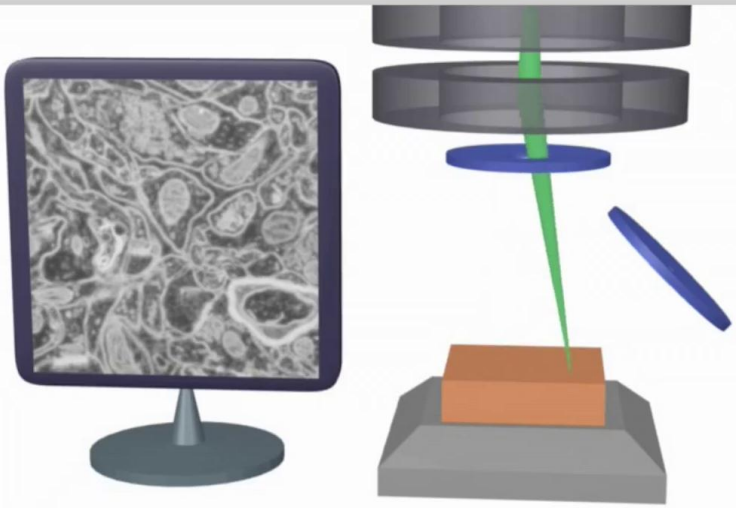
Of course what we could do is get serial images from the electron microscope and gather lots more information from this type of material. And here we have a series of images through a piece of mouse cerebral cortex, and we can start to appreciate the three dimensionality of the features that we're looking at. In these serial images, which are 50 nm apart, because they're 50 nm-thick sections we get an appreciation of this very complex architecture in the brain.

Notes

Summary

18m 31s

Scanning electron microscope



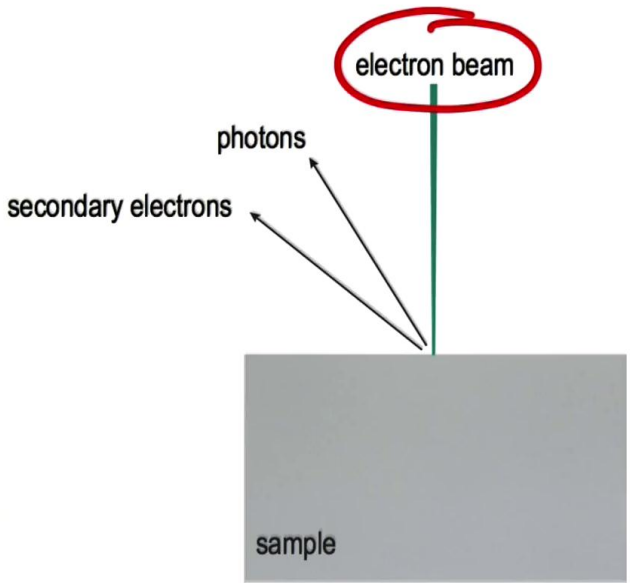
So that was transmission electronic microscopy, and for the last few minutes of this video I would like to introduce you to scanning electron microscopy, because for certain aspects of imaging volumes of biological material, the scanning electron microscope has really taken center stage. Now the scanning electron microscope has, as before, we have an electron gun producing our electrons, that then pass through the condenser-lens system. This then goes through, this very fine beam of electrons goes through these scanning coils, that then is able to put the beam, and scan the beam across the sample. Electrons that are produced are then captured by different detectors to produce our image. So if we look at just a very simple schematic diagram of how this takes place, we have a scanning beam that passes over the sample, and this builds up an image from the electrons that are produced off the surface of the sample.

Notes

Summary

19m 04s

Scanning electron microscope



Cellular Mechanisms of Brain Function

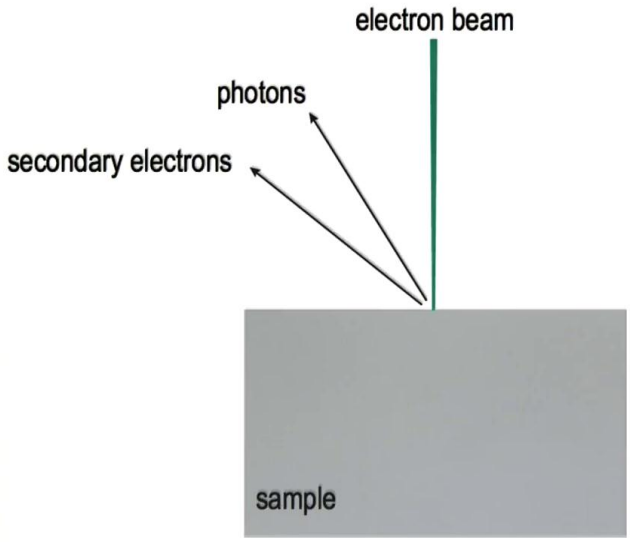
So if we have a look at producing an image in the scanning electron microscope we have the incident electron beam that hits the sample, and then this beam can then produce electrons, so secondary electrons can be ejected from the surface as well as other particles, for example photons.

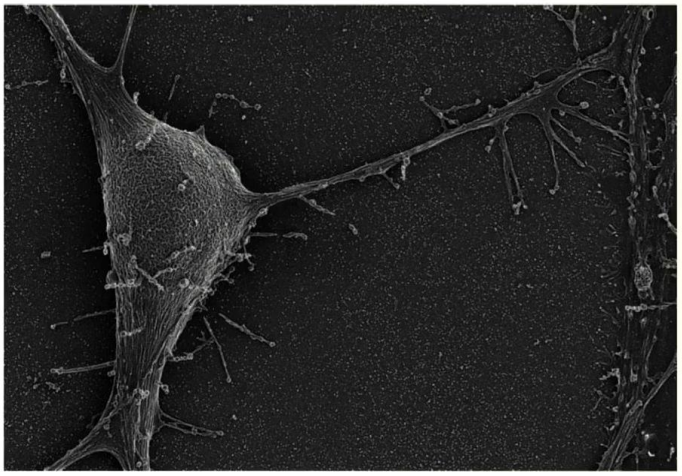
Notes

Summary

20m 03s

Scanning electron microscope





Cellular Mechanisms of Brain Function

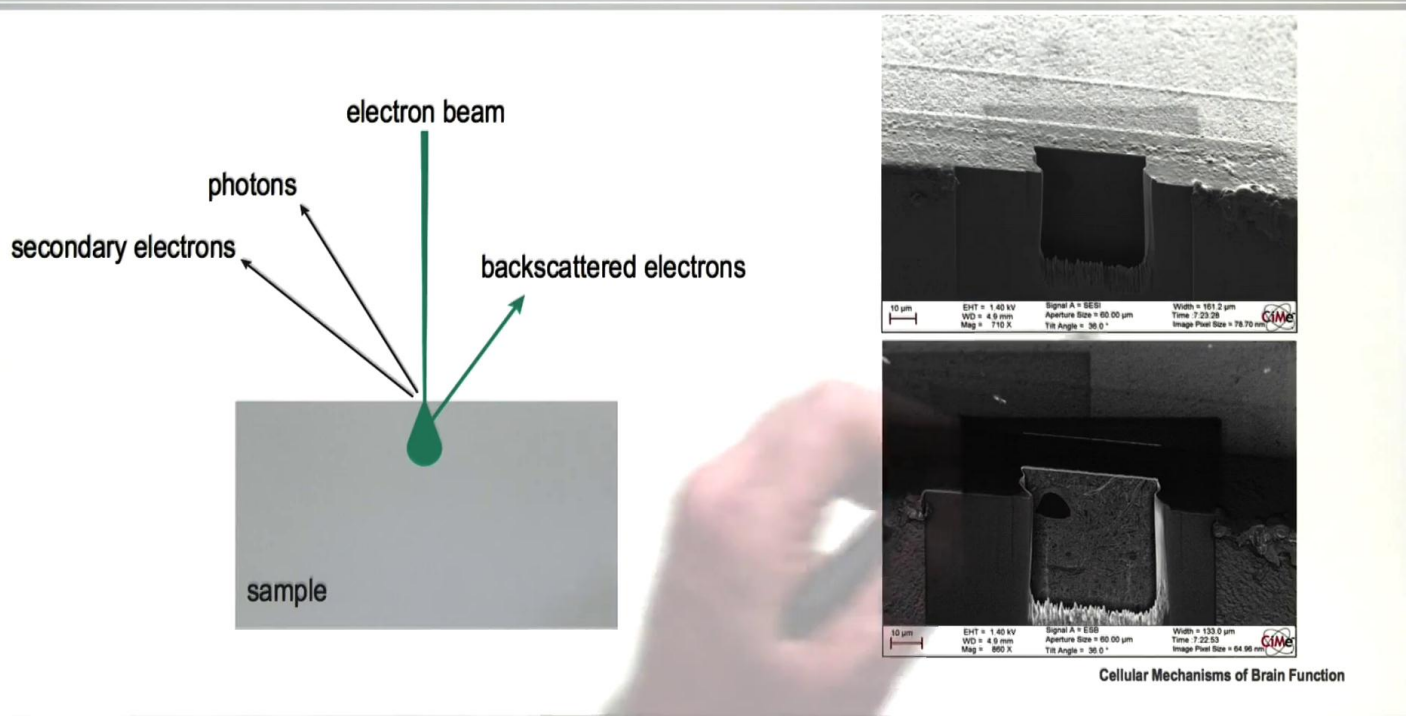
And of course we're all very aware of the type of image we can produce with the secondary electron. So here we have an image of a neuron that's in culture, so it's sitting on the surface of the culture dish, and we can see its general shape, and we can see this process, and some of the smaller processes in the protrude. So this is giving us information about the surface of this neuron.

Notes

Summary

20m 25s

Scanning electron microscope



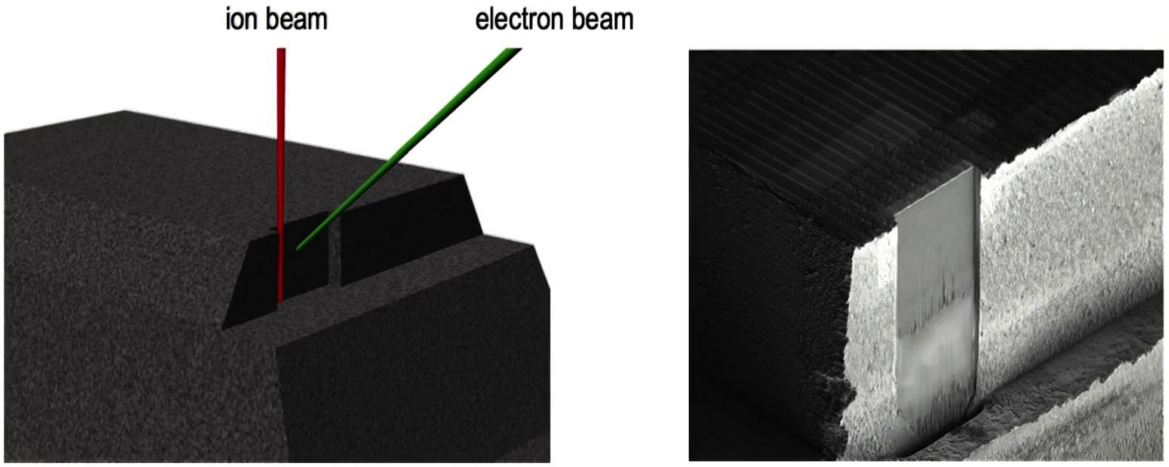
So this electron beam, that is instant on this sample, as well as producing secondary electrons and photons, can also actually interact with the sample, and the electrons themselves can be reflected out. These are backscattered electrons. Now the yield, the numbers of backscattered electrons really depends on the nature of the sample. And the denser and the higher concentration of heavy atoms means we get higher numbers of backscattered electrons. So if we have a sample here with lots of osmium uranyl-acid, uranium atoms and lead, we'll get lots of backscattered electrons, so very heavily stained samples will give a high yield, whereas if we just have empty resins, so in the cytoplasm, where there's very little staining, we will get very few backscattered electrons. In this next series of images, in the top image we have a secondary electron image of the side of the edge of a block of resin-embedded brain tissue, and we can see the surface structure. And in this little window here we don't really see very much, we see the surface. In the second image we have the backscattered electron image, and in this particular window here we can start to see some of the structures within the block itself.

Notes

Summary

20m 51s

Block face scanning electron microscope



Focussed ion beam scanning electron microscope

Cellular Mechanisms of Brain Function

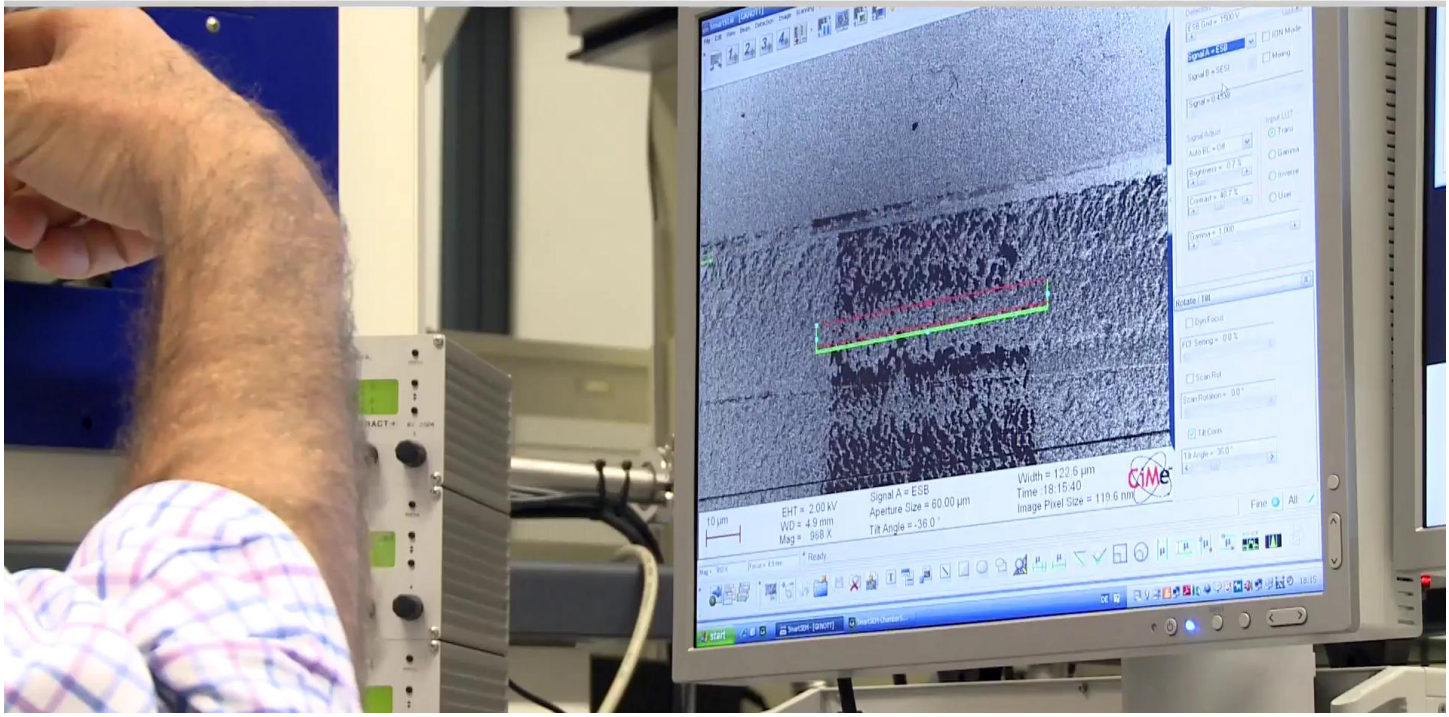
From here we can just about make out what looks like a capillary. So this electron beam that's able to collect images from the face of the sample can then be combined with an ion beam that's able to then polish away just a few nanometer-thick layers from the region that's being imaged. And using a process of sequential imaging, and then ion-beam milling in a focused ion-beam scanning electron microscope, we're able to take serial images through the sample.

Notes

Summary

22m 12s

Block face scanning electron microscope



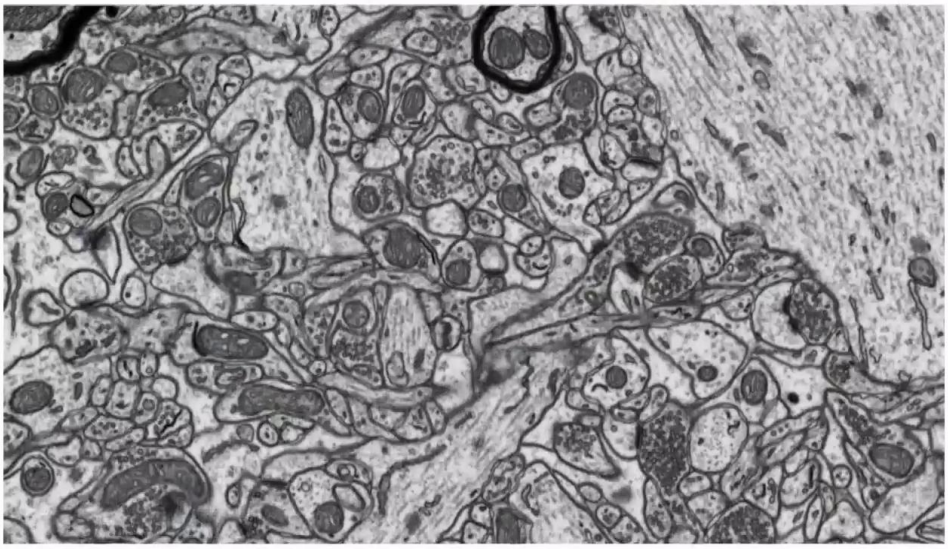
So now I'd like to show you a video of how we use the focused ion-beam scanning electron microscope, or FIBSEM, at the EPFL. Here's the machine, and we first of all take our sample, and load it onto a carrier. You'll notice that the sample has been gold coated to improve conduction. It's then placed into the loading chamber, and then when the vacuum equilibrates, the door opens, and then we're able to slide it in onto the main stage. We can then lock this in place and remove this loading rod, and once that's out of the way we can then close the door of the main chamber, and then start our microscopy. Now through a CCD camera we're able to just see an image of our sample in the microscope. We can then bring it to the right height, just below the pole piece, and then of course we need to very carefully orient the sample at the right angle, so that we're able to image the region that we've preselected. Here you can see a higher resolution image of just the edge of this block that we'll be using the FIB beam to mill away. At a high magnification we see the block edge that's been carefully trimmed so the region of interest is close to this side.

Notes

Summary

22m 45s

Block face scanning electron microscope



Focussed ion beam scanning electron microscopy of brain tissue

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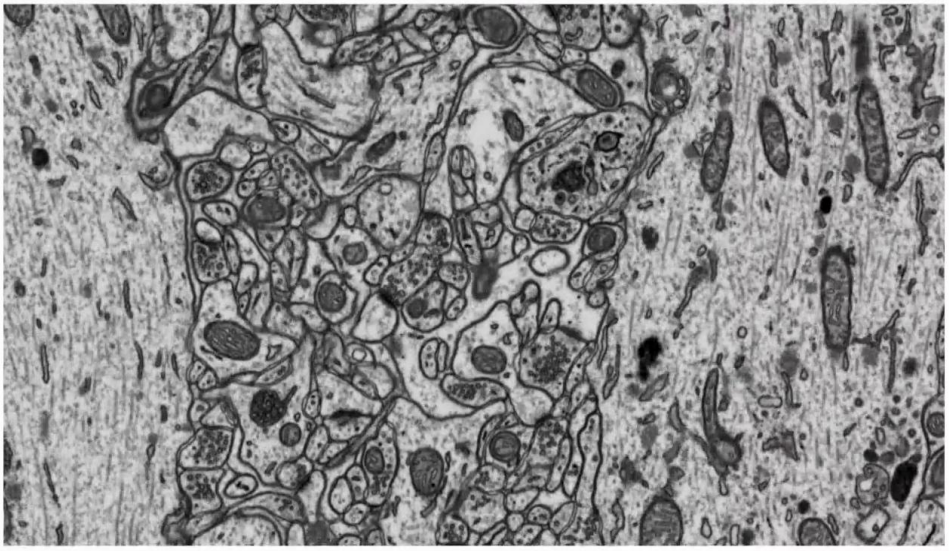
This edge is then correctly oriented so the ion beam's able to scan parallel to the edge that we see here, and as the beam scans parallel to the face it also moves closer and closer, and the thin metal coating on the block surface is slowly milled away, leaving darker regions, which is the resin below. And it's dark because we have few electrons being collected from these areas. So this is the signal from the secondary electrons, but if we change to collect the backscattered signal, we get information from just below the surface of the embedded material, and when the ion beam is being left to polish the face for a few more minutes we're able to see the general tissue structure. And here on the right, and also at the top of the milled face, we can see the dark shapes of the blood capillaries, but also some faint forms of cell bodies in the lower half. At a high magnification we see the heavily stained membranes giving a very strong signal that therefore more electrons are being reflected from them, and in the cytoplasm appearing very dark. We can use this procedure of milling and imaging to get a large series of images through our resin-embedded samples of brain tissue.

Notes

Summary

24m 03s

Block face scanning electron microscope



Focussed ion beam scanning electron microscopy of brain tissue

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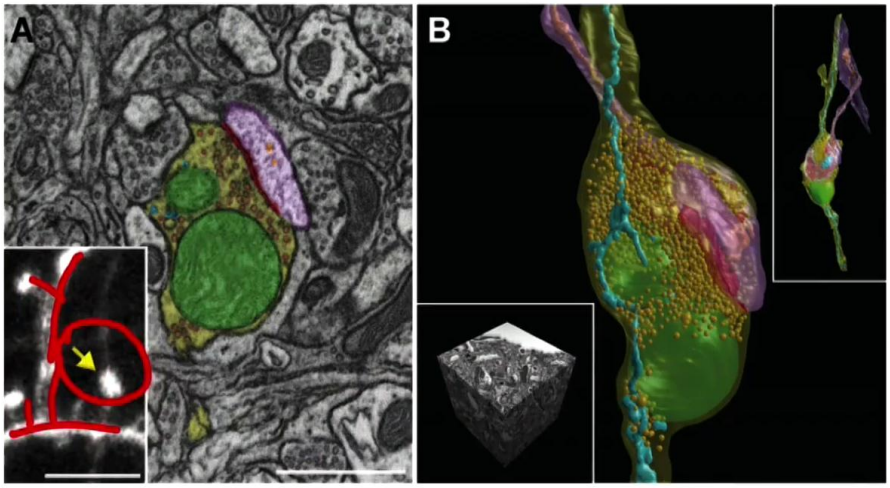
This is, of course, a very automated procedure, whereby the machine will mill away a few nanometers with the ion beam and then switch the electron beam to collect an image. It then returns to milling once more, and this can be repeated many thousands of times to collect a series like the one I'm showing here, that shows images through the CA-1 region of the hippocampus in an adult rat. Here the horizontal field width is about 8 microns, and each of the pixels has a size of 5 nm x 5 nm. Now after each image the ion beam removes about 5 nm of material, so we can collect a series of images, and each of the voxels, or the 3-dimensional pixels, has dimensions of 5 nm x 5 nm x 5 nm. And at this magnification just about all of the stained structures are clearly visible. We have here two large dendrites, one on either side of the field of view, and within them we can follow all the microtubules. We can also see all of the synaptic connections seen in this material, and we're able to identify each of the vesicles within the axonal boutons. So by collecting this quality of images, with these isotropic voxels, we have the opportunity to image large structures at an optimal resolution from which we're able to gather very detailed 3D information.

Notes

Summary

25m 10s

Synaptic ultrastructure



Maco et al, PLoS ONE, 2013

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This then gives us the opportunity to rebuild structures like synapses in very high resolution, in 3D. And here is a couple of images from a figure of a paper that we published recently from the lab, and what we've been able to do is actually take a fluorescent structure that was actually imaged live in the mouse brain, so here we have a 2-photon image of an axon, an axonal bouton, here we have a dendrite, and we can see the dendritic spines protruding. We're able to actually find this back in our stack of serial sections, and then Bohumil Maco in the lab has done this very detailed reconstruction of this axonal bouton, and actually put together this 3-dimensional model of the bouton itself, we have the mitochondria, and then all the synaptic vesicles, the endoplasm reticulum, and of course the postsynaptic structure, the dendritic spine that wraps around this bouton, and we have the synapse in red in between.

Notes

Summary

26m 50s

Summary

I hope from this short video you've been able to understand how we prepare biological material for both imaging and scanning and transmission electron microscopy, and how we can use these techniques to get a very unique view of structures like synaptic contacts. Thank you.

Notes

Summary

28m 02s