Okay. So I thank Dr. Yantian Zhang for the invitation. The last number of years has seen the increased use of model systems, either pathologies or normal states stained with dyes, together with two-proton fluorescent imaging as a way to follow how structures evolve in sort of the normal and diseased states. And there's a wonderful review of this by Thomas Misgeld and Martin Kerschensteiner in Nature Neuroscience Reviews.

What I'm going to talk about today has to do with blood flow in the brain, and my motivation to do this, as you'll see in a moment, is that if you look at the vasculature in the brain, there are sort of three different types of topologies. There are topologies that are very highly interconnected. The pia contains a grid covering the surface of the brain. There are sort of one-dimensional topologies that go from this sort of grid network on the top of cortex that dive into columns, and then there is a mesh of fine capillaries and other micro-vessels below. And just from looking at this and some early studies from a number of labs, including our own stories of the high level fluctuations in the flow, we had some simple ideas that different topologies may be particularly susceptible to different perturbations.

So this works relates both to sort of fundamental aspects of blood flow and to sort of the kinds of damage you might have due to occlusions that you could have in stroke. So what I am going to do in the talk is just review very briefly the kind of measurements we make under normal blood flow dynamics, and then I am going to talk about the use of a variety of optical techniques solely as a tool to induce blockages both on the surface and below the surface in very small focal regions.

Okay, so let me start. Oh, forget the laser. Okay? So the point is that if we look at -- this is a reconstruction of the vasculature within cortex taken by actually filling the vessels with a fluorescein label. And if you look across the surface, there are vessels that commonly -- I'll show you in moment -- make a mesh and we're going to focus on these first. These are called the communicating arterials, but just to keep the topologies in mind, there are vessels that dive down and feed to the upper layers and lower layers and these are called the penetrating arterials. These are pretty much one-dimensional vessels and then there is a large region of sort of sub-cortical microvasculature, which forms very torturous and interconnected set of fine vessels.

So let's just first think about how we might measure flow in these vessels. So we need a contrast agent who we stain the blood plasma with a fluorescent dye. In our case, we have high molecular dextrans, which are labeled as fluorescein. Okay? And then we use two-photon scanning microscopy, technique invented by Winfried Denk in Watt Webb's lab, in order to image into whole animals, and these animals can be maintained for hours under good physiological control. Now, at low resolution we can make very, very large maps. This is a maximal projection showing arteries and veins across the surface, and then from these maps, we can focus on a region of interest and that is shown here.

This is a maximal reconstruction of the microvasculature between about 250 and 350 microns below the surface. You should focus it on the fine vessel here. You should look at a series of snapshots of the fine vessel here. We see bright serum; this is due to the dye. We see dark objects; these are red blood cells that occlude the dye. We then could track the movement of the dark objects and that constitutes a measurement of the speed of the red blood cell. You can also

look at the density of these vessels or we could count the number that pass by. We can measure flux. We can measure speed.

Okay. As a technical issue, we do this in a scanning mode called line scan where we just go back and forth, and this results in raw data that looks like a series of streaks where we're bright while we're in the plasma, we're dark when we hit an occluding red blood cell, and then we're bright again as we go back into the plasma. And as the red blood cell moves, this dark band just moves. We basically have to calculate the slope of this band. Okay? So that we can do in a very automated way, so that constitutes a measurement.

So now, let's go ahead and let's look at flow patterns at these different levels of topology. Okay? So the first thing we're going to do is look at the surface of the brain. We're going to measure flow on the surface the brain. And if we take one of these maps and we trace back what's artery and what's vein, we can actually reconstruct the meshwork on the surface. This is called the communicating arterial network. There has been, I would say, contrasting opinions in the literature as to whether this network in some sense could work as a fault tolerant system. And what that means is that if we were to go ahead and make a blockage at a particular point and then look at flow that is downstream from that blockage, we could ask: does that flow just grind to a halt, okay, or is that flow restored because there's other pathways or other anastomoses that allow that flow to be sustained?

In order to do that, we need to introduce one more technique. We need a way to make a blockage. Now, we're at the surface and we can use the fact that we're at the surface to our

advantage. So we come in with a molecule called a photosensitizer. This is a fancy name for molecules that when it's hit with light will make a free radical and lead to damage of the vessel wall, and ultimately, the creation of a blockage. And because you're at the surface, we could be just at threshold and cause a block at the surface, but as the light begins to defocus and scatter below the vessel, it is now too weak to cause an additional block. So it was a lot of, sort of, histology and published work to justify my claim, but the basic result is shown here.

This is an image, okay, of a frame of microvasculature on the surface. We're going to target the particular branch. This is sort of an interesting branch because we had flow going in this direction. I'm not showing the raw flow data here, but indicating the result of these measurements with an arrow, and then this flow branches in three directions. Okay? We start at this point that targets an occlusion. A little further into our target occlusion, you can see a blockage begin to form. And finally, in this case, after some three minutes, the blockage is complete. There is no more flow, and the critical thing is that if we look at the downstream arm, the minute this blockage is complete, the direction of flow in one of these downstream arms switches. Okay? And the point of it switching is that the direction has changed, but as I'll show you, the magnitude of the velocity, the speed of the blood cell, is on the same order as the original velocity. So it seems to be working as an effective grid.

Okay, so we did this for a very large number of animals. We did this for some 20-odd animals and some 60-odd blockages, and let me just -- these three particular examples. Let me just bring your attention to this top example. Okay? So again, we can characterize the flow not just in one downstream vessel from where we are going to make the block, which is shown by this X, but in

upstream vessels and in a multiplicity of downstream vessels. So we could see how far effects of blockage propagate. Okay? And again, the important point is if I make a blockage at this point, the flow in one of the downstream arms flips. You can see these numbers are within a factor of two. In this case, it happens the post-clot flow is larger. So the point is that the flow is of the same magnitude, and the reason for this is really very straightforward is that if you look at the surface again, we see this propensity for making these closed loops, loops of all different sizes, and that is what gives the surface system its resilience.

Okay, so we can take all of our data, and for each downstream pair of branches, there is one vessel that goes in the same direction and one vessel that is reversed in direction. We can plot the downstream speed. We could also look at potential changes in the diameter of downstream vessels after this block, and in fact, there is virtually no change on average in downstream vessels, which is just a statement that auto-regulation is working correctly in our preparations. And then we can make a plot of the flux, okay, just the volume of red blood cells per units time after the clot versus before the clot. And there is quite a lot of scatter in this data, which in a sense is normal, but the critical thing is that on average -- on average, the flow after the clot is about 60 percent of the flow before the clot, which is well within physiological bounds. Okay, so the bottom line to this data is that this network, this highly interconnected network, communicating arterial network at the surface of the brain acts in a very false tolerant manner. So it's that if you make a single blockage, you get an immediate rearrangement of flow and you get a restoration of flux, which is within a factor of two of the original flux.

Okay. So now, I wanted to move on to the next sort of topology, which is the network that sits below the surface. Okay? So this actually created a challenge, because now we want to address a vessel below the surface without affecting, okay, any of the vasculature above a vessel. So the question is how do we do this with light? And the only way to do this is to use some sort of non-linear interaction so that just at the focus we could actually cause a change by our light but not above or below this focal point. So some ten or 15 years ago, people in the condensed matter community started using light, you know, hot, intense, sharp pulses of light as a means to first generate tunneling of electrons from the molecules that are under illumination, and then, of course, those electrons will get accelerated and form a little plasma. And this can be done in transparent material. It just depends on a non-linear -- a strong enough field to get a non-linear interaction. So sort of a nice example was done in Eric Mazur's lab at Harvard, Applied Physics, where he just went into glass and focused below a cover slip of glass, generated a small plasma, which is this very bright spot, and essentially created a melt. Okay? In his example, this was used to write wave-guides. But for us, we saw this as a technique to address volumes that are on the order of sort of one micron in diameter.

So using this idea, we could focus on vessels below the surface and depending on the power density of the pulses that we put in, it should lead to three effects. So one, of course, is that sufficiently high powers will cause the vessel to burst and this could lead to a hemorrhage. Okay? This is of interest of certain sort of pre-clinical applications, but not for us.

Okay. The second thing we could do is we could focus inside the lumen of a vessel. In effect, this little plasma is going to create a cavitation bubble and this bubble will spread. It will

actually cause the vasculature to expand and this cloud, which is shown here, is a cloud of the fluorescein, which is in the vessel. So we're now causing a vessel to become leaky so that the material could be proteins, in this case dye, that normally never goes into brain tissue transiently enters the tissue. If we continue with additional pulses under this strategy, then we could lead to a block in the vessel. So now what we could do is, we have a tool to study the effect of making a blockage on a vessel -- on the flow in downstream vessels. And we can do this at arbitrary depths below cortex. And again, the key is just using non-linear light to our advantage.

Okay. So we did this and this is again -- this is a two-photon microscope and it's just modified. We followed Margaret Murnane's prescription for building an optical amplifier. These are now available commercially. We introduced the second beam into our microscope, and again, we first make a map, a surface map. We then make a stack of images. We could target a vessel at depth. We're going to target this vessel marked with the yellow X, about 200 microns below the surface. Okay? And then we could -- again, we could map the flow in all these different vessels.

Let me just give one example. Okay? So this is a vessel that, again, that sits about 200 microns below the surface. We're going to sit here and what we're going to do is, we've gone in with a single pulse -- sorry, at this point, this has lead to a small region of damage. There's an interesting phenomenology that very long polymers, 2 million Dalton dextran fluoresceins actually get taken up by cells so they act as markers of the damage site, but after sort of a second pulse, you have a complete blockage of flow. So now, we're going from a situation where we've marked all the speeds before damage and now we've damaged the vessel and we look at the downstream flow. In this case, what we see is here, the immediate downstream flow has almost

ground to zero, okay, unlike what happens on the surface. So again, we can do this for a very large number of vessels.

So just to remind you, if we worked on the surface communicating arterials and looked at the speed after an occlusion versus before an occlusion, the post-speeds are within -- sort of well within factors of two of the initial speeds. Okay? And of course, half the vessels have a reverse flow. If now we look at the sub-surface microvasculature and we make an occlusion at this point, and you look at the flow immediately downstream, as indicated by D1. What we see is that that flow has basically ground to a halt. It has also ground to a halt pretty much two vessels downstream as well. So this is very different, and this is really a statement that the size of the loops of these sort of -- the size of these sort of topological feedback loops at depth is much, much greater than the size of the loops on the surface.

Okay. So there's two things one can do now, so let me just -- so this obviously presented an opportunity to us because although we had not yet finished the full reconstruction of the topology of the vasculature, we do know that there are closed loops at depth. And because of that, we asked, "Is it possible to do some manipulation that actually could restore flow?" And what we wanted to do was manipulate the rheology of the blood. And the simplest way to manipulate the rheology of the blood is just to drop the hematocrit. Okay? And this was sort of an idea that was in the stroke literature but was somewhat complicated in the stroke literature, it seemed, because all the patients that were tested on were somewhat compromised.

So what we did was we made a block and what I'm doing, I'm going to plot the flow one branch downstream, two branches downstream, three branches downstream. Because look, one branch downstream, these points are the initial values of the flow, the baseline flow. And then we make a blockage. Okay? And now I dilute the blood. So one branch downstream, okay, there's very little effect of hemodilution. Two branches downstream, okay, here's my initial baseline value. I make my clot. There's a large drop in flow, but now I dilute. I'm diluting the blood. This is measured by taking samples by -- over a period of a half hour and it ends up with a final dilution of about 20 percent. And in fact, I get a restoration of flow. By three branches downstream, there was very little effect of the clot.

So this is summarized in this graph on top. After a clot, immediately downstream vessels have a huge drop in flow, branches that are two away have a significant drop in flow. But these branches, in fact, get a tremendous recovery. Okay? So this is just a statement in that I've made -- I hate to use this word, but in a sense, the effective viscosity of the blood stream has actually gone down. And this has allowed sort of long anastomoses paths to take over and restore flow at this level.

Okay. So we've talked about what happens on the surface and we've talked about what happens at depth. And we get very different results. And we're going from sort of a complete two-dimensional network on the surface to an interconnected network whose dimensionality we're not sure of yet, but it's somewhere between one and two dimensions below the surface.

What happens now in these penetrating arterials that take blood supply from the top and supply it into the microvasculature? Okay, so we're going to go back to our original setup and our use of photosensitizers. So let me just remind you, if you look down at the vasculature -- look down to the vasculature on the surface of a brain, you see little branches that come off the surface arterials and end in what look like dots. And these little dots are actually plunging arteriols that go down. Okay? An idea then was to -- in order to look at this little segment and again make a blockage at this little segment here. And the question we asked was, first, does this vessel really function as sort of a one-dimensional supply? Is there a region of cortex, which is completely dependant on a single penetrating artery for its supply? Or in fact, is there substantial collateral flow -- substantial anastomoses from related penetrating arterials as shown by these dots? Okay?

So we did these experiments. We'd make a clot on the surface and we'd measure flow at very many vessels below the surface. These experiments were fraught with somewhat more potential for systematic errors due to just, sort of, scattering of the light we were using to make these blocks. So the point I want to make is this, is that there are vessels that are very close to the blockage, such as vessel one shown here, where after we made our laser block, in fact, the flow remained quite steady. Okay? On the average what happens, however, is that vessels that are close to our block have flow -- that sort of -- that immediately dropped to essentially zero. Okay. So the fact that there's some flow -- this and a large body of histology showed us that, in fact, the light is not causing unexplained, unexpected damage. Okay? But in fact, blockage of this vessel will lead to a drop in flow over a large distance.

Let me show you this here. Okay? So we looked at this in two ways, one of which was to make, again, this blockage in the penetrating arterial and we could look at various branches of different orders, you know, one branch away, two branches away, three branches away downstream. The places, if we looked at the fraction of baseline flow as we go from nearest neighbor, next nearest neighbor, et cetera, is very, very little flow. It's a really -- this is a catastrophic block and this really tells us that you really had this picture that the penetrating arteriole is just dominating the supply of blood into its territory below the surface. In fact, because of that, we actually changed strategy and we just started moving away -- we'd make a blockage and we'd move away in distance, okay, from that point to the point where actually you couldn't sort of trace the order anymore because micro-vessels would dive down below our ability to track them. But the point is that if you looked at the fraction of baseline flow as a function of distance from the target penetrating arterial, you see that it drops very close to zero. Close to the penetrating arterial. And then it recovered with a spatial scale on the order of about 300 microns.

Okay. So what I've showed you so far is that we could look -- the brain is cut -- the blood supply to the brain is special because the topology or the dimensionality, if you will, of the vasculature changes depending whether you're on the surface -- it sort of looks like a grid network; whether you're supplying blood to deep into the brain -- would look like a one-dimensional network; or whether you have this deep microvasculature. So we have this set of surface arterial network, penetrating arterials that go down and deep microvasculature.

And as a way to probe the effect of a block, we developed a number of optical techniques to make a block at a single point and then measure the parameters of the flow in the vicinity both

upstream and downstream of the point. And the key thing that happens is that if we have a blockage on the surface vessels, the fraction of baseline flow downstream to that clot is not so very much affected and we get -- you know, two branches or three branches away, there's almost no effect at all. But at the other extreme, if we block one of the penetrating arterioles, in fact, which is shown here by these red traces, there's almost a -- there's a tremendous effect downstream to a very, very high order. And this should -- our belief is that this -- that blockage of a penetrating arteriole will lead to sort of large scale ischemia and this is consistent, okay, though not a proof of, this called -- this sort of pial blockages that people see in MRI scans in human patient populations.

Okay. So I just want to conclude that what we'd like to do is to put this notion -- this relationship between topology and blockages, on hard ground. So I've talked about dynamic experiments and what we're also doing in our laboratory is making a quantitative reconstruction of the vascular network. Now, beyond our blood flow measurements, our intellectual interest, this should have some impact on interpreting fMRI images. But for our case, we'd really like to get sort of a statistical basis of the histology, and maybe not surprisingly, if you look at the surface, they are dominated by these triads. But what exactly is happening at depth is a matter of work in progress.

Okay. So let me just explain who's done what. So, the work on the surface network was the post-doctoral work of Chris Schaffer, who's now an assistant professor at Cornell. The work at depth of the microvasculature was the thesis work of Nozomi Nishimura, who's now a post-doc at Cornell. And the reconstruction of vasculature is the work of -- post-thesis work and the

continuing work as a research scientist, of Phil Tsai in my laboratory. Pat Lyden and Beth Friedman are our close collaborators in the School of Medicine. And I take this opportunity to thank the NIBIB and the NCRR for their support that allowed us to do this work. Thank you.