Now it’s a pleasure to introduce Charles Lin from the Wilmer Lab as Harvard Medical School talk about live imaging of the regenerative micro environment.

DR. CHARLES LIN: Okay, I think the advantage with being the last speaker is that I get to take as long as I want, correct?

[LAUGHTER]

DR. LIN: Ralph this morning gave a wonderful overview of imaging. So I changed my talk a little bit. I’m just going to focus on our work with David Skadden, my primary collaborator. He walked into my lab one day and asked the question can you look through bone, and that basically changed our research direction for the next ten years and it has been a wonderful journey.

Okay, so as you all know, adult stem cells live in specialized microenvironments called stem cell niches, and here are some of the examples – hair follicles stem cell niche, intestinal crib stem cell niche. We’re going to focus on bone marrow because the bone marrow is a major source of both the hemotopoietic stem cells and monozygotic stem cells. And there is a long history of successful clinical translation of bone marrow transplantation or hemotopoietic stem cell transplantation.

And these cells the hemotopoietic glandular cells are really well characterized. Their function and their surface marker have been extensively worked out over the years. Nevertheless, we have absolutely no idea how this

functional organization correlates with structural organization. So we have no idea of how these cells speaking of network of those cell interaction, we have no idea how these cells are organized inside the bone marrow, okay. People hypothesize that there is a hemotopoietic stem cell niche. But how are the more mature cells organized around them. We really don’t know.

So we want to use imaging – live imaging to answer some of these questions. What are the elements of the hemotopoietic stem cell niche, how are they organized and more dynamic question is how do they change under stress or in disease setting. And for transplantation, do the cells go through rinase they have to in order to function. Do they survive, proliferate and differentiate.

Okay, so how do we look into the bone? The part of the bone we’re interested in is a narrow space that’s underneath a critical bone in the tronchanter area, and they look like swiss cheese. So many, many holes. The size of these cavities are a few hundred microns, and each cavity is packed with hemotopoietic cells, maybe 100,000 cells packed into each one of these cavities, okay. So we want to look through the bone into this and then bone is highly scattering tissue. So how we need is confocal or two photon techniques to allow us to optical section. So even though the bone is highly scattering, there are a few ballistic photons that go straight in beneath the bone and then come back out that can be detected. So this is a very

old technology from M. Minsky’s patent from 1961 as a confocal and neurophoton technology. I don’t need to explain that.

So we chose to build our own microscope from scratch, and this microscope and these are Israel and Daniel put in their lifetime building this. The specific feature of this microscope is that it’s fast. It scans at video rate, and it’s sensitive, multi-channel so there are three confocal channels and three two-focal channels. So we can simultaneously capture any three channels at video rate or all six channels together at half a video rate, and it’s customizable so we can adapt this to specific imaging geometry. We can come with additional laser beams for manipulation for doing things like beyond just observing but affecting the tissue.

Okay, so this is all in real time now. We can – there’s a skin imaging. We can look at the structural element by the second homonic generation and look at cells using recorders poson probes and look at flow. This is a real time cell stem of cells in the blood circulation.

So now image through bone. It turns out that there is only one kind of bone we can image through. That’s the flat bone of the skull because it’s thin enough. If you just remove the skin, the bone remains intact. So we look through the entire bone, and you can see this red marrow space even just by eye. So the thickness of the mouse skull bone and the thickest part is only half a millimeter. So in

our imaging that through bone is about 200 microns. So we go just about halfway through the bone into the bone marrow space, and we assume the other half that we don't see is the same as the top half. That's all we can do now.

This technique was first developed by Willy von Andrean at Harvard, and I sort of refined this technique. So this is now looking through the bone the blue here is always the second homonic signal. That's a collagen. We don't need to stain the collagen. It gives you the second homonic signal, endogenous signal. And then the green here are the osteoplasis. They make bone, and the red is a vascular stain by quantum dot. So this looking between 50 and 150 microns below the bone.

Okay, so right away how we can see is the bone marrow vascular density is extremely high, okay. So if we quantify those three-dimensional distance of every green pixel to every – the closest vasculature – the red pixel, this is the statistics. So every green cell is within 25 micron of a blood vessel – 95 percent of the green cells.

Okay, so previously people have used non-imaging techniques to show that there is a vascular niche and there is an osteoblastic niche, and they're sort of separated. And the cells may migrate from vascular niche to the osteoblastic niche. This may be a cryosine place. This may be an active pluriphagen place. We want to observe that. But from this right away it's apparent that the blood vessel comes immediately adjacent to the osteoblastic

niche. So there’s no separation from the osteoblastic blood vessel.

And I’m going to talk about oxygen in a little bit. So there’s also postulated that there’s an oxygen gradient and the hematopietic stem cells, the cryosine cells live in this hypoxic osteoblastic niche. So that’s a question. If the tissue is so highly vascularized, how is it possible that it’s a hypoxic niche. I’ll come back to that.

Now we do stem cell transplantation, and now we add this fourth color. So we again it is from the top in, and there is a single transplanted stem cell. There is osteoblast. There’s a blood vessel. There’s a very narrow space in between, and that’s where the stem cell goes.

To give you an idea of tracking these cells, the number of stem cells that we can inject is very low. So it’s not easy finding one of these cells after injection. So if you look at – this is a case where we have culture tumor cells. We inject a million tumor cells, and each dot here is a tumor cell. That’s a million cells injected. With stem cell transplantation, we can typically inject a few thousand cells. So it’s a hundred to a thousand fold lower than this.

So after each injection, we find between five and ten cells per mouse, and that’s what we have to work with – five and ten cells per mouse. This is the correlation between the number of injected cells and the number of cells we find, and we work in this region, okay. So all

those – if we repeat this enough, we can get enough statistics from five to ten cells per mouse, okay.

So Chris Locelso is the fellow who did this, and one thing we can look at now is a sense of proliferation after infusion. So here is a single cell. We can come in at exactly the same location 24 hours later and see that this cell has become four cells. So this is a first image of in vivo stem cell proliferation over this hand scale, and you can see the statistics stay zero. It's mostly single cells, and then day one, day two, we get more doublings and triplets. And also the distance from the osteoblastic niche is increasing over time, suggesting that there may be an organization that as the cells differentiate or proliferate, they move further away from the endocell surface.

To really chart – to really show that those four cells come from that cell, we need a trick because we cannot keep the mouse on stage for 24 hours and see this cell actually proliferate, right. So the trick is photo convergence. So I'm not going to go into the details. But what we can do is we shine light on this cell, change the color of this cell and we can do an experiment where we only change the color of one cell in the mouse, okay, and then 24 hours later we come back and there's only one cell with the correct color in the entire mouse and that cell has become two. So – and this is really not high throughput experiment. It's one cell per mouse. And so our statistics is very low. But this

is what we get. This mouse – this cell becomes two. Sometimes these cells disappear, and we don't know if it is dead or it migrated away. We need to figure this out. Sometimes it becomes free. In this mouse, we converted four separate cells in four area, and again we see this.

Okay, so this is – people may be familiar with photoconvertible proteins. The photoconvertible proteins is very useful for this kind of cell tracking. But as the protein turns over, the color reverts back to the original color. This is an organic dye that we use for cell tracking that we convert and the color stays permanent. It gets diluated as the cell divides. The concentration changes. But the color ratio stays permanent. So we can track these cells for three or four cell divisions.

And another advantage that this is a new infrared dye. So it can actually be used together with a poson protein which can be differentiation marker. So in this case, you know, we convert the cell and then watch as the GOP turns on to show that it's differentiated.

This photoconvertible technique is also very useful for extending introvital microscopy in time and space that Ralph talked about this morning. We want to extend the field of view that we can image over hopefully the entire animal if that's possible, and we want to extend in time as well. We have experiments where we've converted cells in one location and then watched them migrate into a different location. So that's a trick that we can do to extend our

field of view in space and also in time without keeping the animal on stage for a very long time. So this work is done by Georgie Fugisaki and Avasure Carson.

Now Georgie went on to do another thing. So since we can follow these cells over time, we can now look at cell survival or rejection. So it’s well known that transplantation of hematopoietic cells, you need to have matched donors. So if you transplant allogenetic cells, they’ll be rejected. So we should be able to image this process. And if you are thinking about cell-based therapy, eventually you have to deal with this problem of immune rejection.

So we’ve been working to see the rejection of the hematopoietic stem cells and to our great surprise, we saw that transplanted allogeneic HICs survive for 30 days without immunosuppressive therapy. They sit there, and we can come back 30 days later and we see these cells, okay. This is very, very surprising. The survival of these cells is specific to the progenitor in stem cells. So if we label the stem cells with one color and label the differentiating cells with a different color and inject them both into bone marrow, we see many more of these green cells because we can prepare a lot more of the differentiated cells. Very few stem cells. But after seven days, the green cells are rejected. Very few red cells survive.

In the cynogeneic case, there’s no rejection. So both green and red survive. So this shows us only the primitive

cells are protected, and the rejection takes place at the mature cell stage. And that’s why these cell survival has not been seen before because there are very few cells and traditional method cannot detect these cells, okay.

What is the mechanism that leave these cells in the bone marrow with immune rejection. We know that the bone marrow is a very active hematopoietic compartment. It’s an immune active compartment. So why are they not rejected. There was a previous paper by Zoe, and they showed that human bone marrow contained a high frequency of these regular T-cells. These are immunosuppressive subset of T-cells that regulate autoimmunity as well as transplant immunity. And we confirm by facts that the bone marrow indeed contained a high frequency of these regulatory T-cells. And when we image, so that’s what these green cells are. The red cells are the hematopoietic stem cells that we transplant here. The house is a fast pace reo GOP mouse. This mouse the green cells are the regulatory T-cells. It spreads the forsum protein. So these surviving cells seem to be surrounded by the fast pace regulatory T-cells, and these T-cells reside on the endosal surface, on the bone surface. This is the statistics. Each stat is a three-dimensional distance between the cell and the bone surface or between the green cell and red cell. So more than 70 percent of the cells are clustered both near the bone surface and near these protective T-cells. So that we think is a mechanism how these cells are surviving. And if we do

in vivo time lapsed imaging, we can see that these red T-cells actively patrol the bone surface like they're doing immune surveillance of the bone surface.

Now we can image the survival of these cells. Can we show that these surviving cells are actually functional stem cells? This is where we run into imaging limitation. We cannot think of an in vivo imaging method that will tell us that these are functional stem cells. So we are forced to do the traditional transplantation study. We take out these cells, retransplant it into hemogeneic setting where they will proliferate and show that these cells are able to reconstitute the blood multilineage, reconstitute.

What we can do is answer the second question, are T-regs required for these stem cell survival? So again we image the surviving red cells here in the presence of T-cells. If we come in with an anti-25 antibody that depletes the T-cells, okay, this antibody only targets the green cells. But by removing these green cells, we also remove the red cells. That shows that these green cells, the T-reg cells are required for the protection of these red cells. So I'm not going to go through – so this protection is immediate by IL-10.

Okay, I will come back to this and ask a question: How is it possible that such a highly vascularized tissue is hypoxic, and also does this oxygen gradient actually exist between the vascular niche and the endostral niche. So there was a previous measurement using oxygen electrodes.

So sticking an electrode in an invasive way, and it actually shows that the bone marrow on average is hypoxic. This is two and a half percent TO2 inside the bone marrow. That measurement obviously has no spacial resolution and is destructive.

So now we have an optical method of probing local PO2 inside the bone of a living mouse with micrometer spacial resolution. And so we built on top of this microscope a second arm where we can come in and point the laser beam at any particular location and say what's a pO2 at that location. And the way we do pO2 measurement is using phosphorous in its quenching method is injecting a porforin probe. This probe goes into a triplet state. A triplet state has a very long photo amount of time – micro seconds. During their lifetime, it has chance to collide with oxygen. So by measuring the lifetime, the higher the oxygen concentration, the shorter the lifetime. So we can get direct quantitative measure of pO2 using this method.

So this probe is synthesized by -- because the probe itself needs to be protected. So this benchener protects – regulates oxygen diffusion and protects it from non-specific binding with other components and then is pegulated to make it biocompatible. There are also antennae molecules because this porphorin is a pore photon observer. So in order to do two full photo citation, we put in or he put in these tumoring molecules that actually absorb the

photon and then by thread transfer the imaging to the fourth one.

Okay, so with this, now we measure first the periosteo vessels. So these are the vessels on the outside of the bone, and we see this is a range of pO2 five to eight percent. This is the same range as the recently published paper of green vasculature by David Boas's group, NGH. Okay, now we measure blood vessels in the cortical bone. So these vessels go through bone, and pO2 is already lower. Now as they go into the bone marrow, the pO2 is even lower. And there are few vessels that we can actually follow that your dye's into the bone marrow, and we can see outside the marrow, inside the marrow to maybe separate it by 50 microns. As soon as these vessels dive into the bone marrow, the pO2 drops.

So that explains why even though the vessel density is very high the pO2 is low because the vascular supply already carries a very low pO2. So if we look more carefully at these vasculature, the bone marrow vasculature is actually very heterogeneous. So it's not all cynosoluble vasculature. We can – the most cross grain separation of these blood vessels is into Nestin-positive blood vessels and Nestin-negative cynosoluble blood vessels. So this is a Nestin GOP mouse that we see the Nestin-positive vessels and they are more artery like. So they have higher pO2. The cynosides are more veno-cycle. So they have lower pO2.

Surprisingly the Nestin-positive vessels are closer to the bone, and te cynosides are further away. So this gradient – remember, most people will predict that there's a gradient in going – the bone surface is the most hypoxic. So the gradient will go this way. We actually didn't observe that gradient. If anything, the bone surface is slightly more oxygenated than the cynosides, and that's because these vessels are more arteries and these vessels are more veins. So we did not – so here's our conclusion here. Bone marrow is indeed hypoxic because the vasculature is the pO2 drops very quickly as it enters the marrow. But we did not see that gradient as predictive.

Okay. I want to give equal weights to MSE because I talked all about HSC. So here's my single slide of MSE. This is engineered bone graft. This is what was done in collaboration with Jay Raconti's group, and of course bone graft where a sediment MSC and human vascular endothelial cells. The cuvex are red, and the mazacome stem cells are green. So this is ex vivo before transplantation. We can see that they form tubes already and the MSCs hook the blood vessels. And after transplantation in vivo, we can see that these vessels connect with the host vessel and there's indeed blood flow. Okay.

Where do I want to take in vivo cell tracking? Before we started doing bone imaging, our lab actually focused on eye imaging. So this is the exact opposite of bone. This is the most transparent of organs, and bone is the least

transparent of organs. So with a wonderful optical instrument like the eye, we can just look using a confocal microscope that uses the eye itself as an objective lens. So it’s a microscope without a lens, and we can see the retinal blood vessel, cells in the retina. These are microglia. In the cells, you can see the bentrides of microglia and we can see the blood flow.

Because of non-invasive imaging into the eye, we can actually track this same mouse over 120 days, okay. So this mouse received bone marrow transplantation, and the donor cells are red. So over time we see the green cells disappear and the red donor cells come in and replace the green cells. So this is what I mean by extending introvial microscopy in space and time. We want to be able to stretch our observation over the process that we’re interested in which is often disease progression and which can take weeks, months or even years. So we want to be able to do this. But then at any particular point, we want to come in, zoom in both in space and time and see things happening in real time. So this is a video rate of cell flow and lupasi-endothelial interaction.

So that’s in a transplanted organ like the eye, for scattering tissue we need to improve the imaging depth, and Chris too at Cornell has been pushing this two photomicroscope or even three photomicroscopy or even longer and longer wave lengths. So now he can go more than a millimeter into the brain tissue.

So we're working with him to get these laser sources. And eventually beyond what we can do with even two or three photomicroscopy, we have to use endoscope like Ralph was talking about this morning. This is a rigid endoscope developed by Michael Eakin.

Okay, so I want to stop there and thank everybody in the group as well as the collaborators who did the work.

[APPLAUSE]

DR. LIN: Yeah, please.

MS: Have you done the functional experiment whether the hypoxic versus the not so hypoxic niches are functionally different? Like can you see that cells do divide in the non-hypoxic niche but maybe are quiescent and do not divide in your hypoxic niche?

DR. LIN: Yeah, that would be our goal. This is very recent, though. It's not published. So it's about one month out there. So this is all we have. What we would like to do is in addition to cell proliferation more short term type of function assay. For example, show a time lapse imaging of T-cell migration in this. So what is the effect of oxygen on the regular T-cell migration, and does that have anything to do with the new suppressive function of T regs. That's the first thing we want to do. Yeah.

MS: Maybe I can do a second question if nobody else. The repopulation in the retina is somewhat of the microlial cells is somewhat in contrast to what has been recently reported for the brain by Mir Marad, right. Do you think

that that’s a difference because it’s not in the brain, or so she said that after birth there are local progenitors whereas you saw that your bone marrow progenitors repopulate the T-cells, right.

DR. LIN: Right. So there should be no turnover of both the brain and the retinal micro data. The turnover actually comes from the procedure of bone marrow transplantation. So in order to do bone marrow transplantation, we irradiate the animal. The radiation cause the inflammation that causes the resin microbial to move out and the bone marrow derived cells to move in. So this is actually the effect of the radiation.