DR. MATTHIAS NAHRENDORF: I'm the last speaker before lunch, so I hope that I won't get a tough engineering question from Al because he wants to buy lunch for David Kaplan.

I'm going to talk about optical and fusion approaches and especially fluorescence tomography, because this has really been a technique that has been extremely helpful to follow biological processes in vivo. And I think that's what we're witnessing right now. Even for the folks that say they're not imaging people, they do use optical images. Just think flow cytometry or fluorescence microscopy.

And I think the same penetration is about to happen for in vivo imaging. And one tool that has been extremely helpful for us is fluorescence molecular tomography, and the reason why it stands apart is because it's quantitative. Most fluorescence imaging techniques – at least in vivo – are not quite as quantitative, but this approach really is.

What we're looking at over here is a quantitative map of fluorescence concentration in a mouse. And just to

briefly talk about how this is done, there's a trans illumination set up where the laser goes through 80 different positions underneath the mouse, and you capture the photons that are emitted by fluorochromes inside that mouse by a CCD camera. And this is also calibrated for scattering and absorption, and these data pairs are fed into a reconstruction algorithm that takes into account how photons travel in optically dense tissue.

We have compared optical tomography to a gold standard with respect to quantitative imaging – PET imaging over here – by using nanoparticles that have fluorochromes on their surface, but also PET isotopes such as copper 64 and F-18. And you see that there's really a very nice correlation, if you look at these phantoms containing nanoparticle dilution curves.

What we really see is that FMT optical imaging can be as quantitative as PET, but it's much faster. You can do this in five minutes. It's not radioactive. It's not quite as sensitive, but it works for most of our applications.

There’s one disadvantage or one shortcoming that’s very similar to PET imaging, and that’s limited spatial information. If you look at the image, you don’t know where this blob of signal is coming from. To overcome this we’ve learned a lesson from PET and developed a system where we use anatomic modalities and fuse these datasets by putting the mouse into a translucent imaging cartridge that has fiducial markers on its frame.

You can see these markers by FMT imaging and also on CT here, and then you plug these two datasets into a DICOM reader, such as Osirix, and we have developed a software plugin that fuses FMT and CT datasets and tells you that our fluorescence is coming from the aortic root of ApoE knockout mice with atherosclerosis.

We’ve used this technique in a number of experiments to investigate heart failure, and I want to talk a little bit about infarct healing which is occurring shortly after myocardial infarction and sets the stage whether a patient gets heart failure or not. And what we’ve done here is we imaged the protease activity in

the infarct five days after coronary ligation in mice. This is a protease sensor that is inactive when you inject it. You're looking at a polylysine backbone with fluorochromes on short, side branches. If that backbone gets into contact with proteases that are involved in tissue remodeling and wound healing, they liberate the fluorochromes, and you can excite them, and the photons that you receive report on protease activity.

We used this technique to test the hypothesis that infarct healing is actually different in individuals with atherosclerosis. Why is this important? If you look at most of our heart failure studies, we induce myocardial infarction in healthy mice that are teenagers, so to speak. That never happens in real life because you have to have atherosclerosis to get an infarct in the first place. And the very cells that drive atherosclerosis are also centrally involved in infarct healing. Monocytes that give rise to plaque macrophages are also invading the wound after myocardial infarction.

So, we thought, “What if, after myocardial infarction in a mouse that has atherosclerosis and a higher level of these inflammatory monocytes in the system – let’s look at the recruitment. Maybe it’s higher. Maybe it’s prolonged.” And what we wanted to look at is protease activity, because these cells have high payloads of protease. This could disturb resolution of inflammation, collagen synthesis in the scar, infarct expansion and lead to heart failure.

And we chose to look at protease activity around day 5 after after myocardial infarction. This is a time when non-classical monocytes, which resemble M2-type macrophages, dominate the wound. What we’ve done here is we looked meticulously every day after myocardial infarction and profiled the infarct by flow cytometry. We found there’s a phase one dominated by Ly6C high monocytes. These are inflammatory cells recruited by CCR2. This is the demolition crew that takes out the junk. These cells digest the tissue, and they’re very inflammatory. They have high protease activity. And then later on, the good guys (Ly6C low monocytes) come in and repair the tissue. They stimulate collagen synthesis and angiogenesis.

We imaged around day five that should be really a time when we look at resolution of inflammation. Here are the data, and the upper panel is an infarct in wild type mice. And we can image in two FMT channels at the same time, a protease reporter and also a nanoparticle that's phagocytosed by macrophages, so it reports on phagocytic activity. And we found that in mice with atherosclerosis, we actually have a higher activity in both channels. You have higher molecular signals, and that's also true if you look ex vivo, if you take out the heart and do fluorescence reflectance imaging. You see that the infarct here is really bright in both channels and even more so in the apoE knockout mice.

The beauty of the technique is that you don't have to kill your mouse. We designed a study where we did MRI on day one after myocardial infarction to make sure that there's no difference in infarct size, because that's a co-founder for development of heart failure. And then we did FMT-CT, found increased protease activity and increased phagocytic activity in mice

with pre-existing atherosclerosis, which we think reflects a disturbed resolution of inflammation.

And then we went back to the same cohorts on day 21 to measure end diastolic volume. How big is the heart? How much did it dilate? And we found that this disturbed resolution of inflammation translated into exaggerated LV dilation and more heart failure.

We also turned this around and looked at what myocardial infarction does to atherosclerosis, and the reasoning behind this were epidemiologic data. It's really interesting if you look into what happens to a patient that has a first infarct. There's a 20 percent likelihood to get a second infarct within one year. That's really quite high. Also, there's a 50 percent likelihood that the patient runs into some sort of trouble in form of ischemic complications.

And you could think that this is just because atherosclerosis is progressing, and we now get to a stage where complications start to happen, but we tested here the alternate hypothesis that myocardial infarction does something to the underlying disease.

In this situation, we're looking at acute inflammation on top of chronic inflammation, and the same monocytes are involved in both processes.

We used FMT-CT, so this is really the meat of this experiment. We did protease imaging on week 0 and week 3, and we found some increase in our apoE knockout mice that's shown here, which reflects natural progression of atherosclerosis.

Now, if you induce myocardial infarction right after the first imaging session, you find that on week three in this cohort, protease activity in plaques is much higher. So, atherosclerotic disease is accelerated. These plaques are more vulnerable because proteases digest extra cellular matrix and make it more likely that the plaque pops and triggers an ischemic event.

Because the main cells that provide proteases are monocytes and macrophages, we did flow cytometry on the excised aortas, and we found that, if you just focus on this quadrant here, these are Ly6C high and F4/80 low monocytes, the inflammatory monocytes. If you infarct the mouse, this happens. So, the number

of inflammatory cells, also macrophages, if you compare this, really is much higher.

And we fitted the number of these cells in atherosclerotic plaque over time, we did these experiments up to three months after myocardial infarction. The slope of this function is much steeper in mice that received myocardial infarction, and that was corroborated by other data. Histology showed larger plaques, more inflamed plaques, larger necrotic cores, thinner fibrous caps.

What we think what happens here is that pain, anxiety and the heart failure after MI activates the sympathetic nervous system, which we know signals through a Beta 3 receptor on niche cells in the bone marrow. The bone marrow then releases hematopoietic stem cells, which seed in the spleen and boost extracmedullar production of monocytes. And these are the cells that then can enter plaque and transform the plaque into a more vulnerable phenotype.

This study here pushes the envelope with respect of multimodality. We're looking at mice in which we

grafted colon carcinoma cells subcutaneously, and imaged in a total of five channels. So, we're looking at three spectrally resolved FMT channels which we fused on CT for anatomy, and then also a PET channel. And this approach makes sense because very often, you want to look at more than one biomarker.

In this particular case, we're looking at an integrin sensor that reports on angiogenesis, a nanoparticle that reports on tumor-associated macrophages and protease activity. And our PET channel, for the purpose of cross-validation, also shows you our nanoparticle because it's derivatized with both, a fluorochrome and a PET isotope.

And down here, you see that the signals are coming from different portions of this expanded tumor. So, you can easily think about an experiment where you swap one of these sensors for a fluorochrome that reports on implanted cells, or implanted materials, where you can now look at the microenvironment and changes in the phenotype and so forth.

Histology helps you afterwards to really pinpoint to the source of your signal. And you see that this is quite divergent in this example. Tumor-associated macrophages – these are some blood vessels and protease activity.

I want to discuss an experiment that was led by our collaborators at MIT, Christian Kastrup and Dan Anderson, where we looked into coming up with alternative therapies for vulnerable plaques, because we think that we're about there, that we can detect these vulnerable plaques in patients; but what we do not have is a good answer as what to do next. What do we do to these patients? Some people think you should put a stent into a lesion like that. That's pretty aggressive, because there are side effects to stenting. And very often, these lesions are not stenotic.

So, could we come up with a system where we deliver local therapy without injuring the vessel? And so what Christian and Dan did here [was] they searched for a material that can be applied to the endothelium inside the vasculature. They call this "vascular

paint.” And what they did is they learned a lesson from muscles, which use a catechol to stick to underwater surfaces in the ocean. And they did some in vitro experiments, where they checked if the material – the gel that they came up with – resists shear stress, and that’s the case. And we took this now in vivo and used imaging to look at how this material fares inside carotid arteries.

So, we’re looking at the carotid artery of apoE knockout mice with atherosclerosis. The gel was painted on here, and there’s an infrared fluorochrome incorporated into the gel, so you can see it by intravital microscopy. And we also injected an intravascular agent so you can tell that the blood flow is still there.

We went back to the same area and see that the gel was still there up to 30 days after apllication of the vascular paint and you can, here, see a microscopic image of how the gel lines the blood vessel.

As proof of principle, we then incorporated dexamethasone, which is a very potent anti-

inflammatory drug, into this gel. And first we looked into delivery into the vascular wall using a dye. You can see that this gel delivers this dye into the vascular tissue. And now, if you deliver dexamethasone to atherosclerotic plaque in apoE knockouts, you find that in these treated artery segments, there's a thicker fibrous cap, less macrophages and inflammatory markers such as transcription factor – [unintelligible] – MMP9, which is a protease that digests the fibrous cap, are down regulated.

I have one more vignette here that shows you delivery of materials. This is drug delivery incorporated in nanoparticles. So, these are lipidoid nanoparticles that are designed to augment silencing siRNA therapy. And one really big problem in this area is the delivery of siRNA to the site of action, and we used imaging to follow our siRNA. The idea behind this was to silence CCR2. This is a chemokine receptor that is specifically responsible for recruiting inflammatory monocytes.

What we could do here is not take a sledgehammer to the immune system, but really only target these cells and leave non-inflammatory monocytes, or repair-oriented macrophages, or lymphocytes, or sessile immune cells, alone. And the idea is that once you knock down this receptor inflammatory monocytes can't travel towards MCP1, which is secreted at the site of inflammation.

Again, what we did here is FMT-CT imaging – dynamic FMT-CT imaging – to follow the biodistribtution of fluorescently labeled siRNA encapsulated into our nanoparticles. And you see early on, there's a blood pool signal that goes away quickly. You can actually fit the blood pool signal which shows that the blood half-life of our materials is eight minutes. And then you see that the signal comes up in the spleen. This is also true if you open up the mouse, you see that the spleen is the brightest organ.

And this got us really excited, because we found that the spleen contains a large reservoir of Ly6Chigh monocytes. So, you see these clusters here in the red pulp? These are inflammatory monocytes. In a steady

state, they’re quiescent; but if you induce myocardial infarction, they become active, increase their velocity. They enter a splenic vessel here. You can see this cell entering the vessel and then taking off. Then the cells travel to the infarct, and if you take out the spleen – and this is FMT-MR imaging here – you see that the signals go down.

So, that’s really good if our nanoparticle makes it to the spleen, but what about the cell - cellular targetings? We co-stained the tissue for CD11b. This is a marker expressed by myeloid cells, and you see that this nicely co-localizes with our fluorescent siRNA.

And then we profiled the spleen by flow cytometry, found really high uptake in all phagocytically active cells – macrophages, to some extent, even B cells. But the brightest cells were our inflammatory monocytes, and this is the knockdown[. On mRNA and on protein level, you see less expression of the CCR2 receptor, also by flow cytometry, less of the protein on the surface. And then if you do a functional assay

– migration assay, you see that their capability to migrate towards MCP-1 is reduced.

And this has a phenotype in vivo, so we induce myocardial infarction here. You're again looking at the Ly6C high monocytes in a mouse that was injected with the control siRNA. So, we digested infarcts and took[?] this profile in here. Now, if you treat them with siCCR2, you see that there's less recruitment of these cells, and that results in a smaller infarct size, if you normalize to the area at risk.

So, for the last couple of minutes, I want to switch to a PET MRI, because this is really something that I find quite exciting – a new, emerging theme where you put together two of the leading modalities in cardiovascular imaging. This is like merging the Mercedes Benz with a BMW.

[CHUCKLING.]

DR. NAHRENDORF: MRI is really leading with respect to soft-tissue characterization, and PET is quantitative and very sensitive. This is our low-cost approach to

this. Ralph mentioned this fiducial vest yesterday, so this is last season's iteration. There's a more up-to-date version of this jacket, which has a bit more fiducial points; but the point really is that you see it by CT. You also see it by MRI, and we also use a bed where you don't have to move the mouse around, so you can really go into one scanner and immediately into the other one; and you end up with nicely fused images. So, this is just FDG in the myocardium.

What you end up with are these type[s] of images. What we do here is we're imaging macrophage content using nanoparticles that are derivatized with a PET isotope, and you see signal in the aortic root; and at the same time, you get really very nice anatomy. You can't do this with CT – very nice functional and time-resolved contrast on the aortic valve. You also can take this into the heart and do heart failure studies. So, up here we're doing the delayed enhancement MR that shows you the infarct. This is something that's standard in the clinic, and it co-localizes early on after myocardial infarction with FDG signal and reflects inflammation.

You can take this a notch further to molecular MRI, so here we used an MPO sensor that reports on inflammation in the heart, and this is a PET sensor that's sensitive to plasma transglutaminase, also an important aspect of infarct healing. And you can then integrate these molecular datasets with functional datasets, for instance MRI tagging that reports on myocardial strain.

This is my last slide that stresses that one really beautiful aspect of imaging can be that you have multi-scale approaches, so you can use in vivo imaging to noninvasively follow your materials or your disease over time, in different cohorts. And then you can look at the organ level. These data are from Claudio[Vinegoni at the Center, who uses OPT, which is a mesoscopic approach where you can look at the entire mouse heart in different fluorescent channels with quite spectacular resolution – different channels. And then you can take a step further and look at the cellular level with microscopy and flow cytometry.

With this, I would like to close and acknowledge the fellows that did all this work: collaborators at the

Center Fil Swirski and Ralph Weissleder, and our funding from NHLBI. Thank you very much.

[APPLAUSE.]

MODERATOR: Matthias would love some questions.

Q: I had a question about the quantification of the in vivo images. We've been doing some similar things with a dual probe that is fluorine NMR, but also has a fluorescence probe that targets inflammatory cells in situ[?]. So, we can see similar kinds of inflammation around, say, a myocardial infarction, or a tumor, or something like that. When we do a correlation between the two, we find that the fluorescent signal, although mostly correlates with the NMR signal, in certain tissues there is actually maybe some intrinsic tissue darkness. So, if we look at the spleen, for instance, the signal doesn't – it doesn't correlate; whereas, in a different type of tissue, we find a strong correlation. I'm wondering if the FMT imaging accounts for the difference [in] darkness in different tissue types.

DR. NAHRENDORF: So, the general question is it does, and it should. There are specific targets that are more difficult than others, so especially if you look in the abdominal cavity. There is quite a bit of autofluorescence coming from, for instance, the feces. So, one important point is that you put your mice on [a] non-fluorescent diet. You're probably doing this already, but this may screw up your signal.

So, I think if you look at where did these calibrations – the calibration curve that I showed there was a phantom[?]. If you go in vivo – we've done this in the subcutaneous tumors also – it doesn't look as nice, but it's still .8 or something. But these subcutaneous implanted tumors, they're also fairly easy.

Now, if you go into the spleen, you might have gut that overlays it, so this might be where some of your differences may come from.

Q: Well, actually, we've done some of this, actually, ex vivo as well. So, we've taken the organs out and put them on a light box, and new can still see the

difference between between, say, a spleen and the liver in terms of the correlation.

DR. NEHRENDORF: Okay.

Q: So, I was just wondering, you know, if that's something that you've looked at.

DR. NEHRENDORF: Well, there're the obvious things that I'm sure you're taking care of, such as decay and so forth, but it could also be that – you know – fluorochromes come off, that isotopes come off at different rates. That could screw up correlation.

Q: Thanks.

Q: In one of your slides towards the middle of the talk, you had a model for after the first MI. Then there's very often a second MI within a month. And the key mechanistic step was pain anxiety, which later leads to neutrophil release. Is it standard to put patients on anxiolytics, actually, clinically?

DR. NERENDORF: Very much so.

Q: Oh.

DR. NEHRENDORF: So, when patients come into the emergency room with acute MI, they have a crushing pain, and they fear for their life, so they – we put them on opioids and anxiolytics and beta blockers. These are the first things you do.

Q: Okay. Thank you.

DR. NEHRENDORF: But I think, you know, this principle might be fairly general. I don’t know at this point, but I think maybe this is not just limited to myocardial infarction.

MODERATOR: Okay. I think let’s thank – [unintelligible] – and all the people – [unintelligible].

[APPLAUSE.]