On imaging, speed, and the future of lymphocyte signaling

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During a recent roundtable discussion, we captured some personal perspectives on the new insight that advanced imaging techniques promise to bring to the study of lymphocyte signaling. The experts present their views on the power of imaging, the problems that need to be overcome, and the potential of the technology.

Images of cells are perplexing. Genetically tinted by the jellyfish fluorescent protein painter kit, cells of all colors and shapes turn serious academic journals into modern art catalogues. After decades of abstract thinking and frequently fruitless interpretation of bands, dots, and their graphic derivatives, the attention of many scientists is now attracted by the opportunity to see almost everything in "real" life. The increased communication value of images is a part of a rapidly evolving cultural tendency demanding real-time event documentation. Nowhere could be more remote from this problem than the caught-in-time Certosa di Pontignano, an old castle near Siena, Italy. Maybe that is why we decided to organize a roundtable discussion about imaging during the 2004 EMBO workshop on Lymphocyte Antigen Receptor and Coreceptor Signaling held at this location (1). We asked the experts to reflect on the impact of imaging on a field that has been dominated by biochemical approaches. Our aim was to capture some personal viewpoints on the real and virtual power of imaging and the new challenges the technology brings. Can the traditional craft of cell manipulation match the high speeds that can now be visualized? What new reagents are needed to allow us to take full advantage of imaging in the lymphocyte signaling field? Should imaging data be quantified (definitely, yes!) and if so how? (See also the related Commentary on page 501 of this issue [2].) At the end, we asked the participants to fantasize about how we might be using imaging technology to study lymphocyte signaling in the more distant future.

I regret that we could not present all of the lively discussion, but we have included comments and exchanges on some of the key topics that were covered. We thank all the participants for their willingness to share their insight and opinions.

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POWER OF IMAGES

Mark M. Davis: If you ask, "What's the value of imaging?" one important thing is that it allows us to escape the tyranny of population biochemistry. We've been forced to indulge in the fantasy that if you have a million cells in a tube, and you hit them with something, that they're all going to do exactly the same thing at the same time.

Glossary
FRET: fluorescent resonance energy transfer
FRAP: fluorescence recovery after photobleaching
ZAP-70: ζ-associated protein of 70 KD; a pivotal molecule in membrane proximal T cell receptor signaling
Immune synapse: organized structure that forms at the interface of an antigen-presenting cell and antigen-specific T cell
Lipid rafts: cholesterol-rich plasma membrane microdomains that have been implicated in lymphocyte signaling

However, we know that a lot of stochastic processes are going on. If you just grind the cells up at different time points, you're averaging over all those processes. It's only when you can see an individual cell that you can see it was stimulated, and then went on a particular pathway. And there's a level of quantitation we've never seen with biochemistry. We're getting much more precise answers to questions like the amount of calcium that's being released during T cell activation. We can see that one peptide gives you X amount of calcium, and two give you 2X, and so forth (3).

Lawrence E. Samelson: With imaging, it's not just that you're able to overcome the heterogeneity of the cell population; you're able to overcome the heterogeneity of the intracellular molecular populations. Many of the molecules that we tag and look at, in terms of intracellular signaling proteins, exist in different pools in the cell: some are cytoplasmic, some are recruited to the membrane, others are associated with the cytoskeleton. It would be impossible to study these different populations without imaging the individual cells and individual molecules.

Bernard Malissen: We are also feeling out the speed at which signaling processes are working, and it's very fast. Neurobiologists have known that for a very long time. We are just put-

ting our finger on the speed at which a T cell is responding to an antigen. So imaging gives you an idea of the timescale we should be thinking of.

Arthur Weiss: I think that imaging in real time is critical. But in addition, FRAP technology allows us to see things that we never imagined, in terms of the turnover and the dynamics of structures that we think are static. Because you see an immune synapse, you think everything is just sitting there and immobile with no turnover.

MMD: There are a lot of technical challenges involved in asking questions of a detailed molecular nature, of molecules that are within particular cells, at particular times, and in particular places. But it's the future of understanding how these cells work. There are huge challenges in getting markers for events inside the cell. But there are already some that are specifically designed as indicators of a particular kind of phosphorylation. We need many more of those for all the different signals that might be going on.

Rose Zamoyska: Single molecule imaging is coming, and it's not that far off. That really is going to add another dimension to the way we're going to be able to analyze signaling events.

IMAGING MEETS BIOCHEMISTRY AND GENETICS

Oreste Acuto: Nobody would disagree with all that has been said so far. But we should never forget that biochemistry gives us the fastest way to establish protein–protein interactions, and the highest resolution.

BM: Ultimately, genetics should allow you to order a pathway without having help of biophysics. If you have powerful genetics, like in the yeast, you can do very nice ordering and epistatic analyses of pathways, and you don't need imaging.

RZ: One of the problems with biochemistry is that you can never look at the consequences of the signals, because the cells are lysed. And one of the advantages of imaging is that we're developing techniques where you'll be able to look at the differentiation consequences, downstream of particular signals.

Christopher C. Goodnow: Whenever I hear the biochemistry angle, I always think about Arthur Kornberg and how proud he was of DNA polymerase in the tube, until the geneticists knocked it out in bacteria, and they replicated just fine. It's very hard to order pathways and I think the triangulation between biochemistry, genetics, and imaging is going to be essential. You need to know where things are in the cell, and their kinetics and the dynamics, which is what the imaging tells you. You need to know what could happen, which is what the test tube experiment tells you. And then the genetics tells you what is actually needed for a given process. As a community, we don't put enough emphasis, in our publication and referee processes, on when the genetics and the biochemistry are disconnected. For example, we have a ZAP-70 catalytic domain point mutant mouse, which has a severe problem with thymic positive selection (unpublished data). The genetics is telling us that there is a real problem. But when we look at tyrosine phosphorylation and do old-fashioned calcium analysis, we can't see any problem. So the biochemistry is disconnected from the genetics. The question is if you did high speed imaging, would we suddenly be able to connect the genetics with the biochemistry?

Michael Reth: The immune synapse is a nice example of how difficult it can be to correlate image analysis data with the biochemical behavior of molecules inside a cell. When the synapse was first discovered, everybody thought that one had finally found the molecular organization of signaling proteins at the activated T cell antigen receptor. Yet when people later on analyzed synapse formation at different time points and used antiphosphotyrosine antibodies (4), it became clear that TCR signaling happens much earlier than synapse formation, and that the synapse may have more to do with the secretion of cytokines than with the initiation of the TCR signal.

MMD: Absolutely, when we first see things we're invariably going to misinterpret them. Seeing may be believing, but it isn't always understanding.

MR: Another source of artifacts is the use of GFP-tagged proteins to follow the behavior of molecules in a living cell. This is a powerful tool, but it is important to first test whether the fusion to GFP is not drastically changing the localization or biochemical behavior of the fusion proteins inside the cell. Another problem is the use of fluorescent reagents to visualize structures in living cells like lipid rafts. In this respect, it was interesting to see that when FITC-coupled cholera toxin was used to detect raft structures it resulted in raft aggregation (5). So you are creating what you want to observe.

> "There's still a bag of genetic tricks that hasn't really been applied to imaging but which could be used ...photo-activatable GFP, for example, and temperature-sensitive mutations."

QUANTITATING IMAGES

MR: One problem we have to solve is how to quantify our image analysis. When I see a publication with a beautiful picture of a cell showing the localization of the GFP-tagged protein to a particular membrane structure, I wonder how representative this image is of the behavior of these proteins in the cell population. To overcome the bias generated by our eyes and our brain, we need to apply computer programs for image analysis which, similar to a FACScan, can quantify images and order cells according to the image phenotype.

LES: Many of these problems are problems that all cell biologists have. You look at a picture in a journal, and you say, "How do I know this is representative?" That gets into the question of honesty, and ethics, and basically

good science. But programs for quantitation exist; you can track movement; you can quantitate velocity; you can quantitate dynamics. So a lot of what we need is there.

Salvatore Valitutti: It's very possible to quantitate morphology. It's very tough. Very time consuming. But it is possible to ask authors for quantification, or even to show the originals from which the quantitation was made.

Alexander Tarakhovsky: Do you think that there is an absolute necessity to introduce ways to quantify images?

SV: I think it will be in the interests of people doing morphology to somehow enforce measurements of intensity, for instance, at the immune synapse.

MMD: The referees always ask us for quantitation: what does the cell population do? How many cells do this? How many cells do that? On some occasions, when the referees have been skeptical about our interpretation of the images, they ask for all the images, and we send all the images on a CD. So the vetting is actually easier than it used to be, in the sense that you can transmit vast amounts of information very quickly, so that people can be sure that you're not being subjective. As to a program that Bill Gates would sell us to do this—fine, when it happens. But there are many different questions you can ask using imaging. I can imagine a very simple kind of software being good for some kinds of imaging analysis, but completely useless for other kinds.

Claire Hivroz: One of the problems that we have to face is that while these new imaging techniques are really nice, they are sources of artifacts, also. We really need to do the analysis carefully. Biologists are not physicists, and sometimes we don't know how to analyze the image, or we don't know exactly how to use the software. I think we have to make great efforts to work with physicists, to really understand what we are doing because being sure, for example, that what we are looking at by FRET is really an interaction between two proteins is not that easy.

ADVANCING THE TECHNOLOGY

MR: By using GFP-tagged proteins we can employ imaging to localize proteins to certain subcellular structures, but what is really lacking are more techniques to not only measure the localization, but also the activity of a protein. Of course, we can measure the intracellular increase of calcium by fluorescent dyes, but these dyes cannot be localized to certain places inside the cell. What we need are more GFP variants that change their fluorescence upon modification, like calcium binding, phosphorylation, or oxidation. With such techniques, you can then see in real time, not only the localization of molecules in the cell, but also at what time point these proteins become active or modified.

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Anjana Rao: I'm dealing with a program of transcriptional regulation that is sensitive to very low intracellular calcium levels—lower than can be measured reliably by the available technology. So I think, in the case of calcium imaging, we're clearly going to need some new technology in the picture.

MMD: There's still a bag of genetic tricks that hasn't really been applied to imaging but which could be used. There is development of photo-activatable GFP, for example, and temperature-sensitive mutations. So it might be possible to give cells a burst of a laser, for example, to alter the function of molecules that you had modified.

AT: Do you think that one can manipulate structures such as the immune synapse?

CCG: The strength of genetics is in the specificity of the dissection. But the limitation is that you just have no temporal control or spatial control. So, in that respect, it's a very limited tool. The ideal scenario would be to replace genetics with pharmacology. To have small molecules that you can add in to inhibit signaling pathways, which are as specific as genetic manipulations. But, of course, where we get into trouble is in knowing how specific a compound is. This is where the neurobiologists, and the cardiologists, have it over us as immunologists. They've got a panoply of quite specific agents to dissect processes such as cardiac contractility.

AT: Do you think that genetic manipulation could allow conditional protein inactivation at a given time point?

CCG: Inactivation has to happen very fast. The only thing would be some adaptation of photo-inactivatable or temperature-sensitive alleles, which as Mark [MMD] has pointed out, could allow you to eliminate a wild-type signaling protein both rapidly and in a specific region of a cell.

MR: New in vivo imaging studies can follow the dynamic behavior of single lymphocytes in the tissue of a living mouse. This, in combination with genetics, will allow us to understand better the dynamics of cellular interactions inside the immune system. To follow the behavior and activity of single molecules inside a lymphocyte is much more demanding technically, as these are rapid and very dynamic processes. One needs machines with a very fast kinetic image acquisition if one wants to follow these events in real time. Furthermore, if one wants to track single fluorescent molecules, one has to use a lot of laser light to detect them, and this could be another potential source of artifacts. Laser light not only results in photobleaching, but can also generate reactive oxygen species. As many signaling processes inside the cell are highly sensitive to redox changes, it is possible that shining a lot of laser light on the molecules can change their regulation and behavior.

AW: One thing I want to stress again is quantitation. And maybe genetics can

help there. Chris [CCG] talked about a point mutation which doesn't show any biochemical difference. We might not be able to validate that our GFP constructs are behaving normally using simple overexpression systems. I would argue that maybe we can use genetic approaches to test the constructs we use to validate the fact that we're expressing the right amount of protein, in the right place, at the right time, and that those proteins are regulated properly. Maybe we can harness the power of genetics for proper expression—right place, right time, as well as right level.

AR: In terms of technology development, there are two things that I would focus on. Microinjection of mutant proteins into cells that don't have that protein would be a very good thing to develop. The protein can be tagged in various ways, with small fluorescent probes for instance. And second, I think electron microscopy will be important. Think about the work that Gunther Blobel did, for instance, with the nuclear pore and with nuclear import and export. He had the biochemistry. He had all the different subunits. And then he used EM to localize them in the basket of the nuclear pore. The stable immune synapse is such a structure that could benefit very greatly from EM analysis.

IMAGINING THE FUTURE OF IMAGING

AT: Now I want you to dream.

AR: To dream?

AT: Yes. So, without discussing limitations, tell me how do you think in ten years your dreams will be fulfilled?

[Laughter]

MR: My dream is a very powerful computer program that is able to generate a virtual cell where we can test our hypothesis of protein signals inside cells. Using the primary sequence data, this program should be able to generate the tertiary structure of the protein not only in its frozen (crystal) stage, but also its normal dynamic stage in solution. With such a program, one could follow the conformational changes of proteins and better understand the dynamic processes of protein–protein interactions during cell signaling. So that is a dream, but I think one has to wait quite a while for such a program.

MMD: I don't think a computer would be able to know how evolution built a cell. So I think that dream is going to come to a bad end. But in my childhood, there was a movie called "Fantastic Voyage," where there was a tiny, little ship that was built, and miniaturized humans were in it. They cruised around the bloodstream, and they looked at the lymphocytes and other things from this point of view. So a good dream would be one where you miniaturize the ship even more, such that you could go into a cell, through a channel or something, and cruise around the cell and watch different bits of the machinery moving up and down, or doing whatever it does.

AR: I think you'd find what Schrödinger predicted in 1944 (6). You'd find probabilistic movements. I think the whole workings of the cell are governed by probability and stochastic movements that, in turn, are governed by the affinity of interaction. My dream would be to apply the imaging techniques we're talking about at the membrane, today, to find out what happens in the nucleus.

LES: Mine is another cautionary tale from early twentieth-century physics, and that is the Heisenberg Uncertainty Principle. We have to watch out for what we do to these systems. Certainly by manipulating systems we create artifacts. So that's a nightmare.

[Laughter]

OA: My ultimate dream is that we abolish competition. We create a big center and attack very fundamental, basic paradigms in a few systems. It is not strictly necessary to know everything, with everybody working in their own little corner. Why not combine genetics, imaging, and biochemistry in a dedicated center where scientists can perform challenging experiments, like physicists do? That's a dream. That's something that I think should work.

[Applause]

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