

## FOCUS: MICROSCOPY AND IMAGING

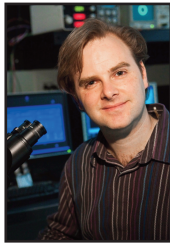
# Pushing the Boundaries: The Development of Super-Resolution Microscopy at Yale and Beyond

## An Interview with Derek Toomre, PhD

Rachel K. Zwick\* and Barbara A. Schmidt

*Yale University, Department of Molecular, Cellular, and Developmental Biology, New Haven, Connecticut*

Derek Toomre, PhD, is an Associate Professor at Yale University in the Department of Cell Biology. He established and directs the Yale CINEMA (Cellular Imaging using New Microscopy Approaches†) lab, which is a state-of-the-art live cell imaging center. He received his PhD from the University of California, San Diego, working on a biochemistry and glycobiology project in which he generated new fluorescent probes and used extensive chromatography but very little microscopy.



Toomre

During his postdoctoral fellowship in the renowned lab of Dr. Kai Simons, however, he advantageously applied new techniques from the rapidly developing fields of microscopy and live imaging to his research in membrane traffic.

Throughout his career, Dr. Toomre has made numerous contributions to the development and widespread use of microscopy techniques, especially Total Internal Reflection Fluorescence (TIRF) Microscopy. His lab uses these microscopes not only to visualize the processes of endocytosis and exocytosis, which control how proteins enter and exit cells, but by using “optogenetics” to acutely control cell activity with

---

\*To whom all correspondence should be addressed: Rachel Zwick, Yale University, Department of Molecular, Cellular, and Developmental Biology, Kline Biology Tower, 219 Prospect St., New Haven, CT 06520; Tele: 203-432-3482; Email: rachel.zwick@yale.edu.

†Abbreviations: CINEMA, Cellular Imaging using New Microscopy Approaches; TIRF, Total Internal Reflection Fluorescence; FRAP, Fluorescence Recovery After Photobleaching; PALM, Photo-Activated Localization Microscopy; STORM, Stochastic Optical Reconstruction Microscopy; SIM, Structural Illumination Microscopy; TIR, Total Internal Reflection; RNAi, RNA interference; EMBL, European Molecular Biology Laboratory.

Keywords: TIRF, microscopy, super-resolution microscopy

light. His research has implications for a range of diseases spanning from type 2 diabetes to ciliopathies to cancer.

Here, we talk with Dr. Toomre about how he became interested in microscopy and the work that he and his lab have been doing with TIRF microscopes and optogenetics. He gives credit to the biologists, physicists, and individuals in industry with whom he has closely collaborated and tells us how he is able to cultivate new collaborations within the CINEMA lab.

***You are currently at the forefront of the development and application of state-of-the-art microscopy, both in your lab's research and as the director of the core imaging facility at Yale, the CINEMA lab. What drove your interest in advancing the current state of microscopy?***

My interest in microscopy stemmed out of initial frustration and failure. Namely, I went into Kai Simon's lab on a biochemical project to isolate vesicles in an *in vitro* assay. I worked on that for about 6 months, and I struggled and failed. So it was in failure that we readjusted and thought maybe we need to approach this differently. Maybe it is just too hard to make this two-stage *in vitro* assay work. And that's when I decided to go into microscopy.

I was really lucky to be at the right place and at an exciting time. The GFP era really got going while I was working toward my PhD. I was actually lucky enough to have classes with Roger Tsien, who got the Nobel Prize for his discoveries and developments with fluorescent proteins. It was clear that GFP gave a different opportunity, a different window, for looking at cells. That's when everything shifted and became more focused on microscopy and imaging approaches. And that's when the live cell imaging opened up and the tools were changing.

Personally, I was really interested in seeing exocytosis, or seeing vesicles fuse at the plasma membrane. Although by this standard fluorescent microscopy (with live cell imaging) we could see these vesicles appear and stay for a while and then suddenly

disappear, it was not very satisfying. Seeing something just *disappear* doesn't really tell you that much. It wasn't clear if it was just going out of focus or if it was doing what we were hoping that it was doing, which was fusing with the plasma membrane. I think there are some things that are led by frustration.

So just around that same time, Dr. Wolf Almers, a Max Plank director in Heidelberg, had made these new advances with a technique called Total Internal Reflection Fluorescence Microscopy, or TIRF microscopy. At that time, around year 2000, all TIRF microscopes were homebuilt systems, and the one that he put together took his postdoc student about 2 years to make. I begged him, "Could we possibly come down and do an experiment?" The answer was initially no (I now understand the need to vet projects), but I managed to convince him. I came down for one afternoon, and within a couple of hours of working with his postdoc, Jürgen Steyer, we had these amazing movies.

We saw these fusion events: little explosions, kind of like looking from the city above. It was like seeing a box exploding at night. You could call them cellular fireworks, and you could see these events very clearly. So now it was clear that we got something. We knew that we were in good shape.

Then, about 3 days later, we got some bad news. Wolf Almers took a new position and was moving to Portland, Oregon, and they were already packing up their microscopes. So with this microscope that had taken a really long time to set up, we had [obtained] nice data, but not enough for a publication. Jürgen was going to be there for a while, and we decided to make a different type of TIRF microscope, called a prism TIRF microscope.

A lot of instruments were left behind [by Almers' lab]: lasers and microscopes in pieces. So I worked with people at the European Molecular Biology Laboratory (EMBL) instrument labs who had a very nice machine shop, and we basically built all the pieces that we needed to make the thing work. It was a bit of a learning curve to see how to assemble it. It took about 6 months. I think if you are

determined and get help, you can figure out a lot of things, and I like tinkering. I like optimizing things and making instruments work. We got [the TIRF microscope] working again and could recapitulate our results and do more experiments.

***There are only a few TIRF systems available worldwide. How were you able to realize the commercial potential of this kind of system?***

Based on that instrument, I had my first discussions with industry. A company called TILL Photonics realized the amazing potential of this sort of system for imaging and was one of the first to make a commercial system. Soon after, Olympus followed. It has become much more of an instrument to which a lot of people can have access, so it has really changed the field. Although the first TIRF microscope was invented by Dan Axlerod in the mid-1980s, there was a really long period, basically 15 years, where there were no commercial systems.

Once we had the commercial systems, we still had problems. Part of the problem was that we had interference fringing — artifacts that are prevalent in almost all of the commercial systems. We thought about a new way of getting rid of these aberrations and also how to make it work better with multiple penetration depths. This is where we could work in an interdisciplinary way. I worked with a physicist, Robert Roorda, who is very solid in optical design and implementation, to build a prototype system which we thought might work to get rid of these patterns. It uses what we call conical lenses, which are similar to a zoom system in a camera. We built a prototype in the machine shop, and it was all made of plastic, just to see if the basic concept was working. It wasn't even a microscope, rather it was just a test illumination system on an optical rail.

The prototypes were good and promising, so we started building a system for a microscope. We — Rob and then another physicist, Dr. Vladimir Polejaev — were able to further develop this system with support from the New Innovator Award that I got from the NIH director.

***Where is the final product now?***

We built two instruments. The first instrument worked well, but the only problem was that it was a mechanical device and could not be changed quickly. We saw an opportunity to do it more quickly and that became basically the next instrument. And then we saw another opportunity to do more with FRAP (Fluorescence Recovery After Photobleaching), which is really important for studying cell dynamics, and we were able to incorporate that.

In all these cases, it is a series of steps. Everything I've done here has started with a problem. What was very satisfying after coming up with it and seeing things working well was to actually be able to see it realized in a real product. It is one thing to think of something that could be a patent: It has to be new, useful, and non-obvious. But it doesn't actually have to be made. It doesn't have to have a prototype. But to get a company interested in seeing it, producing it, they want to see it is actually working. That's what we were able to do. We interacted with several companies, and that itself is a long process, but we are very happy to see it being realized by a company called Applied Precision Instruments, which was then acquired by GE Healthcare, and it is quite nice to see it come the complete circle and be made and used by others.

***Now that we've heard about its creation, could you give a technical overview of TIRF?***

Sure, TIRF works because you generate an evanescent field in aqueous medium, which selectively illuminates only a thin optical section of the cell. Wide-field fluorescence microscopy lights up the entire cell. Confocal microscopy uses a pinhole to reject light, so light that is out of focus gets rejected. That can be from any plane. TIRF microscopy is different. It doesn't reject light with a pinhole. Rather, it selectively illuminates only a thin region on the bottom of the cell, in close proximity to the cover glass.

It is useful to think of an analogy. Total Internal Reflection (TIR) happens when you have two mediums of different refractive index, such as glass and water or water and

air. One place you can see total internal reflection is at the bottom of a swimming pool. Sitting underneath the water, looking straight up, you see the overhead lights, but looking at a glazing angle across the pool, you see the reflection of underwater tiles in the side of the pool. Another place you can see total internal reflection is a diamond. Because it is able to reflect most light back due to its high refractive index, it is able to reflect more light back than would, let's say, glass. This causes it to have its characteristic sparkle. This also happens in fiber-optics. When you send an optical signal along a fiber-optic cable, as long as it's not bent too much, it keeps bouncing back and forth down the fiber. That's why it is a very effective way of communicating a lot of information at once. For TIRF microscopy, the wave actually penetrates [slightly] into the other medium. This penetration depends on the angle, the wavelength, and the difference of the refractive indices of the two mediums.

The advantage is that you selectively illuminate a thin part of the object without the noise of all the other fluorescence. That is its strength, but also its Achilles' heel. It is very good for imaging things on the surface, but if your process is not near the surface, it is not as good. Now with multi-angle TIRF, you can go a bit deeper into the cell. But you are still dealing with less than one micron — not very deep.

The amazing thing to me about TIRF microscopy is the sensitivity. TIRF microscopy is one of the few techniques in which you can actually detect and see a single molecule easily. You can do that in cases where there'd be a lot more background because you are only going to light up things near the surface. The disadvantage is that you get rid of information that is deeper. So as most of my research is at the plasma membrane and processes nearby, there is no problem with that. The advantages greatly outweigh the limitations.

***Which fields of research are using TIRF well, and which aren't using TIRF microscopy yet, but should?***

You see quite a lot [of TIRF microscopy usage] on membrane traffic and also cy-

toskeleton [research]. I think there are more open areas on signal transduction. There's a lot of signaling that's happening on the plasma membrane. In virology, I think [TIRF] would be quite useful for looking at how pathogens get in and out. There has been some work done in immunology, but probably not as much as there could be.

***TIRF belongs to a set of techniques collectively known as super-resolution microscopy. Could you explain it in this context?***

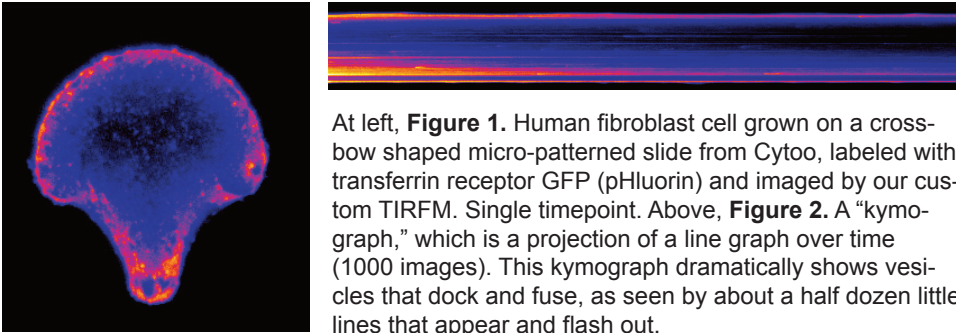
Super-resolution microscopy is a really hot area, and it is a little bit confusing because to certain people it means different things. The simplest way to think of it is that it allows you to see things that either push or bypass the diffraction limit, the limit of what you think you will be able to see.

TIRF microscopy does this in the axial or the Z-axis. It is basically a near-field technique, so you might have an optical section less than 100 nanometers, while normally the limit would be closer to 500 nanometers. The PALM (Photo-Activated Localization Microscopy) and STORM (Stochastic Optical Reconstruction Microscopy)-type super-resolution microscopy works by localizing single molecules.

In all these cases, it is kind of cheating the system. It is not that the laws of physics are wrong or are being disproven; [we are] finding the exception. It's finding the cases where the rule doesn't apply.

The exception in the case of PALM is when you are dealing with objects and you can see [its single molecules selectively], you don't need to resolve them. If you can see individual molecules, you can instead try to localize them. If you can localize them very well, you can determine the center of these soft, blurry objects and instead use that to kind of paint-by-dots.

There's another version [of super-resolution microscopy] called HiLo, which is a little bit like TIRF. With HiLo, the light actually goes into the other medium at a very shallow angle, so it gives a very high contrast. I used to go scuba diving. If you had a light, and you shone it on particles in the water, a lot of signal is reflected back. But



At left, **Figure 1**. Human fibroblast cell grown on a cross-bow shaped micro-patterned slide from Cytoo, labeled with transferrin receptor GFP (pHluorin) and imaged by our custom TIRFM. Single timepoint. Above, **Figure 2**. A “kymograph,” which is a projection of a line graph over time (1000 images). This kymograph dramatically shows vesicles that dock and fuse, as seen by about a half dozen little lines that appear and flash out.

if you are able to illuminate from the sides, like they do in most flash photography underwater, you don’t get as much of that scatter back at you, so you get better contrast. That’s not total internal reflection, but [rather] a high angle of light that increases the contrast. The same microscopes that we use for TIRF also work well for generating this HiLo-type illumination.

Then we have structural illumination microscopy (SIM). Our SIM scope takes up a full small room, but it’s a very powerful system. What is really nice about that system of the super-resolution modalities is that it is the most similar to confocal microscopy. You can take the same specimens with multiple colors and double the resolution in X and Y and Z that you would normally be able to get out of a confocal microscope. It pushes the resolution, it pushes the ability to see, and people forget that what is important in resolution is usually not only in one axis. So although it is only two times in X and two times in Y and two times in Z, the combination of those — the 8-fold increase in resolution — is very powerful.

***What questions are you currently pursuing in your lab with TIRF microscopy, and how do you expect this research to be most beneficial?***

My main focus is on imaging of exocytosis and endocytosis, but particularly of exocytosis (Figures 1 and 2). We’re interested in [several] things that pertain to disease. One is how membrane traffic is dysfunctional in relationship to type 2 diabetes. Working closely with Jonathan Bogan, we’re looking at the Glut-4 transporter, which is important for glucose uptake after insulin [and the resulting vesicle trafficking pathway]. By using

TIRF microscopy in combination with new probes and image analysis, we can distinguish between two different sizes of vesicles, which respond to insulin in two distinct ways. We could see vesicles that were smaller than the diffraction limit, so 50 nm vesicles, and we could distinguish them from 150 nm vesicles. In so doing, we could see that there were two different pathways that responded differently in time and space to insulin. Now we are following up on that work.

In a paper with Felix Rivera-Molina, a senior research scientist in my lab, we were [also] able to see the exocyst, which is a tethering complex used in vesicle fusion, for the first time. So remember that 10 years ago we saw vesicles go static for a while and then fuse? Well, this is the protein complex that is important for holding on to these vesicles and making them go static before fusion. We would never see this without TIRF, because it is a dim signal, [and] TIRF is good at seeing things with a low signal-to-background [ratio].

And we’re doing a lot on the molecular side about having certain molecules labeled, controlling things with light, with what’s called optogenetics, which is a really hot area.

***Could you describe optogenetics?***

Optogenetics is an area that is mainly seen in the neuroscience community. It is a way to use genetically encoded proteins that can respond to light. They’ve engineered a way to use probes, not only to see, as we typically do in fluorescence microscopy, but also as a way of controlling activity.

In most of those optogenetic applications, it is used to open up a channel. For instance, the channel rhodopsin. By shining light onto these proteins, you can make the



channel open. You could shine light on a subset of neurons in an intact animal and turn on their activity — fire the neurons. It is reasonable to predict that maybe in the next decade or so, the Nobel Prize will come out of that. So that is one context.

The other context, the one that we are using, is a bit different. We don't use it to control a channel. We've been working closely with Pietro De Camilli's lab to use a light switch to cause proteins to dimerize. That could be interesting as a target-bait system. When you turn the light on, you could recruit a protein, such as a kinase or a phosphatase, to go to a cell surface organelle such as the plasma membrane, where it will then be active.

So [optogenetics] is a very nice way to acutely control cellular activity, which is different from most biological experiments. [One could compare it to] RNAi (RNA interference): the ability to knockdown a protein and see the effect on a pathway, which has been the other revolution in mammalian cells over the last decade. It is very powerful; however, there are caveats. When you remove a critical protein, the cell has a few options. One option is compensation, that is, use another pathway to compensate for the loss. As an analogy: You are going home, and the road is closed. You can either get stuck there or you can go by a different road. It may not be as fast, but you can get there. That compensation by chronic treatment of cells is a problem. The other potential problem is an off-target effect. The analogy would be that the railroad shut down and caused more people to take the highway, and then the highway got clogged. Now, the highway is not clogged because of the specific pathway you are thinking about, but because of something else — a secondary effect. [Additionally], with knockout and knock-in experiments, you might be treating cells for days, or an animal for months. That is a long time frame.

With optogenetics, you can turn things on and off in a second or two. It is pretty amazing to have that control. In this rapid timeframe, there is no chance for compensation, and one can see and study with high spa-

tial-temporal precision the specific pathway of interest. Time is one factor, being that you can switch them on and off in seconds versus potentially days, but you can also do it in a specific spatial region of the cell. One of our microscopes makes it easy to use optogenetics to turn things on in a selective region, to see if the process matters in one place versus another. [We] use light not only to see the [membrane] traffic but also to control it.

***What is specific to the research environment at Yale that has enabled or stimulated your pioneering work in imaging?***

One thing that is really nifty about Yale is that there has been a very strong commitment to imaging. In Cell Biology, it was set up by Dr. George Palade, who got the Nobel Prize 40 years ago for using electron microscopy to look at cellular organisms, their organization, and to identify pathways in cells, including membrane trafficking pathways of secretion. It is hard to imagine the field of Cell Biology without electron microscopy. Knowing the molecules, it's going to be either biochemistry or genetics, but giving them a subcellular spatial context is very much at the very heart of cell biology. Where is it happening in time and space and what are the molecules and machinery and mechanisms? That's why imaging is so critical and is so fundamentally linked to cell biology.

What Yale did in the last decade, which is pretty remarkable, is that it identified light microscopy as one of the key areas [of biology]. There's been incredible work here with MRI and PET centers and imaging. When Dr. Jim Rothman arrived in 2008 [to chair the Cell Biology Department], one of the areas that he identified as a key frontier and was able to push ahead in a very proactive way is super-resolution imaging. As many people would tell you and as reflected in his 2013 Nobel Prize, Jim is very visionary — he identifies a hot area that should open up biology and goes after it. He knows the impact of the right tools and people. We made a number of hires and coordinated with Dr. Jim Duncan to create an imaging center at Yale, which is absolutely incredi-

ble. So what we have is a critical mass of users and instruments together. We have all types of super-resolution microscopes at Yale. We cover the full breadth of it and that involves TIRF microscopy, PALM and STORM, STED microscopy, structured illumination microscopy — with several variants of these microscopes. There is really quite a large breadth and depth there. Dr. Joerg Bewersdorf has done a lot of the instrument development and is really pushing the boundaries of what we can see and do.

***Tell us about the CINEMA lab at Yale: What is it and what is happening there now?***

Our imaging center is about cutting edge techniques. There's one side that's basically an industrial partnership — working very closely with the manufacturers to bring alpha-beta type instruments to [a center] where people can see them and use them. For example, when the spinning-disc confocals were coming out, we worked very closely with the manufacturer to get new cameras and to encourage development of new areas in FRAP and photoactivation.

The other part is instrument development. We've developed a number of microscopes, mainly in the TIRF microscopy space. They are state-of-the-art instruments using technologies that we have developed and that will perform better than standard instruments. The challenge with things that are very new or cutting-edge is that they are more difficult to use sometimes. They may not be as turn-key. We work with users to show them how everything works and bring them up to speed.

One technique we are focusing on is spinning-disc confocal microscopy. The difference there relative to the confocal microscope is live cell imaging. We have it set up with special incubation chambers, and the system itself is extremely fast. So the time that it would take one normal confocal 2D scan, you have now taken the 3D stack with that system. So it's very much geared for the live cell.

The SIM super-resolution microscope system is unique. I believe there are only a couple currently on the East Coast. They are

expensive instruments, but they are extremely powerful. It is very easy to go from regular confocal microscopy to super-resolution imaging, and that is a big advantage because people have to think about how much time they want to commit to super-resolution imaging — making new probes that you may need for PALM and STORM. It is very helpful to first have a good look without going through large hurdles. The other big advantage is that SIM easily works in multicolor (e.g., 3 channels). Working with two or three colors quickly gets very tricky with these other techniques.

In my opinion, these methods all have their tradeoffs, so you have to try different ones and see which is the best. There's not usually going to be one single answer. So we work closely with people to identify what their problem is and then help direct them along the best channel that could be most informative for their research.

***You mentioned two ways in which you have collaborated with industry on microscopy development: Working with manufacturers to bring microscopes to researchers and developing microscopes as a researcher and bringing the new technology to industry for commercialization. Tell us about this.***

It's nice to see when academia and industry can interact positively. They both have their different strengths. People in academia are very good at inventing things, but that doesn't mean that those inventions will be good for others to use as they are. They are sometimes too complex. For example, the SIM system was invented by academic researchers Dr. John Sedat and Dr. Mats Gustafsson. But had it not been licensed and commercialized by Applied Precision Instruments (a GE company), probably no one would be using it because it is just too complex to build. To have an instrument really make a difference, it has to be in the hands of many users. It has to enable biology, and that's what we are really about. We aren't just building instruments because they are really cool instruments. We want them to enable new biology and new discoveries.

***To conclude, what advances can we expect in imaging?***

I can predict that in the next 15 years, there is going to be a Nobel Prize in the fields of optogenetics and super-resolution imaging. It's an extremely exciting time be-

cause we know that we are going to be surprised. We know that we are going to see more.

These changes are going to shape our understanding of cells and how cells function and how they dysfunction in disease.