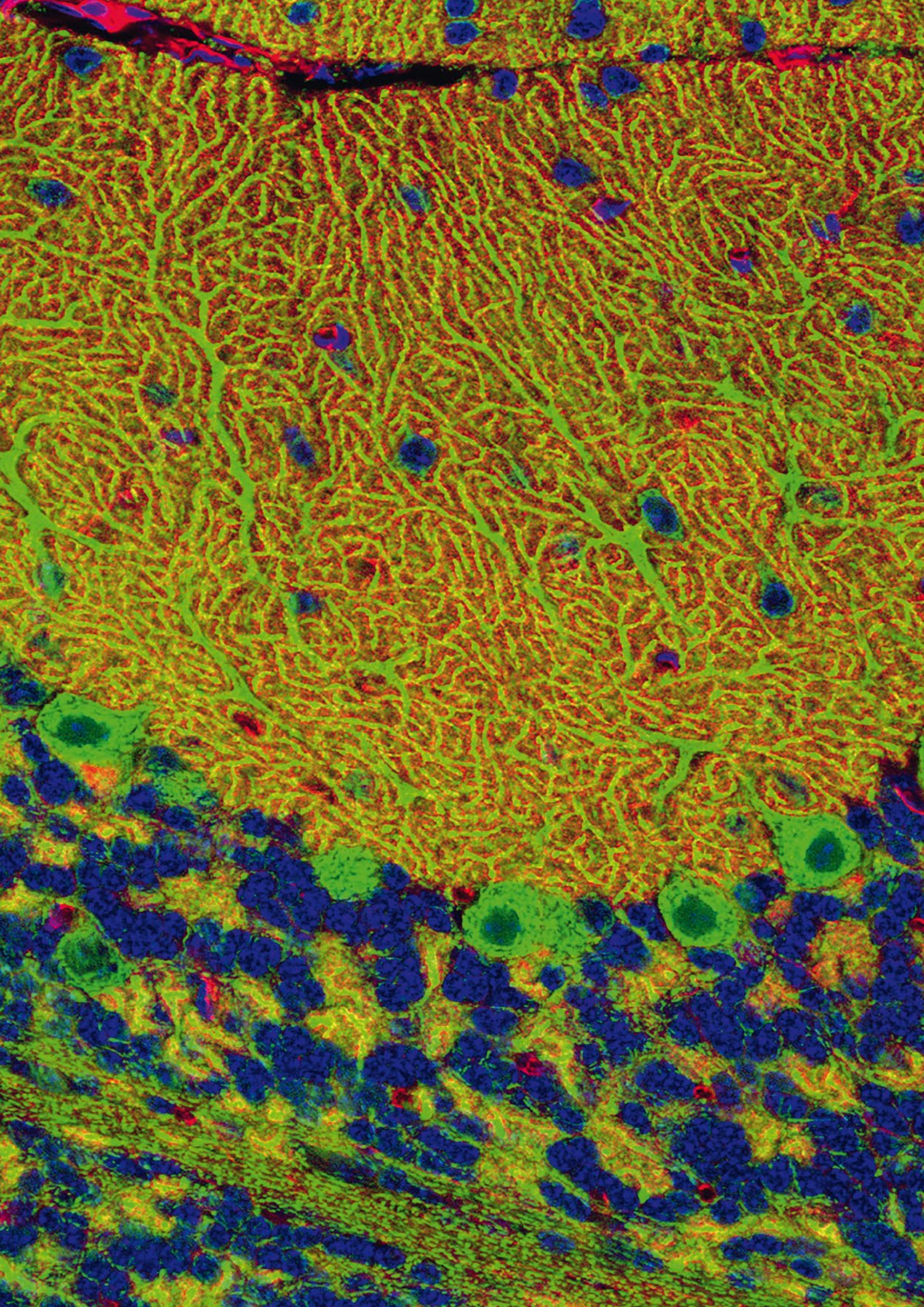


Subcellular Dynamics in Neurons

Research probing mechanisms that organize vital processes in cells, from single-celled fungi to the human nervous system, advances our ability to understand – and potentially to diagnose, treat or cure – some of the most devastating illnesses, such as neurodegenerative diseases. At the same time, it sheds new light on some of the basic mysteries of life.

Hans Fischer Senior Fellow Maya Schuldiner is an Israeli biologist whose main research focus at the Weizmann Institute of Science is on organelles and protein functions in yeast, as revealed through a combination of genetic screening and imaging. Hans Fischer Fellow Melike Lakadamyali, originally from Cyprus, is a physicist in the Physiology Department of the University of Pennsylvania's medical school, where she too is investigating the molecular machinery at work inside cells – in her case, largely by pushing microscopy beyond what was considered possible just a few years ago. Gelsenkirchen native, TUM alumnus, and former Hans Fischer Tenure Track Fellow Thomas Misgeld heads the Institute of Neuronal Cell Biology in the TUM School of Medicine, with close, active ties to the German Center for Neurodegenerative Diseases (DZNE) and the Excellence Cluster SyNergy. His lab uses in vivo *imaging in mice and zebrafish to study what happens inside and between nerve cells in development and disease.*

They have joined forces in the Focus Group Subcellular Dynamics in Neurons to explore how mitochondria and other organelles travel and communicate in the crowded space of a cell – particularly in the neuron, with its extreme geometry and complex functions. How do the principles at work in the simplest cells – governing how cellular components are shuffled around by molecular motors along cytoskeletal tracks, and how the tracks themselves may be regulated – translate to neurons?

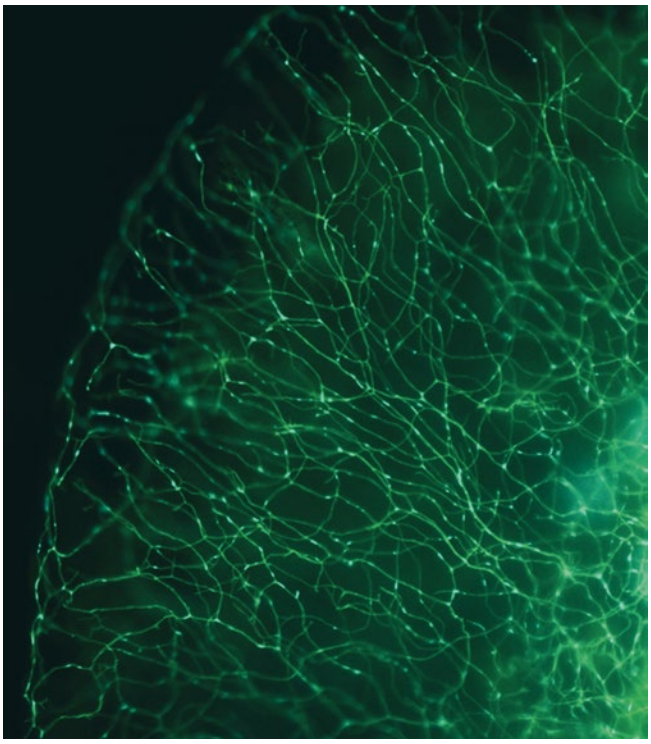
The TUM-IAS conducted an hour-long interview that spanned seven time zones: with Maya Schuldiner (MS) in Rehovot, Thomas Misgeld (TM) in Munich, and Melike Lakadamyali (ML) in Philadelphia.

Left: Purkinje neurons in the mouse cerebellum, where neuronal mitochondria (green), neuronal cell contacts (=synapses; red) and cell nuclei (blue) are labeled. Because synapses are packed with mitochondria, the synapses appear yellow.

Q: Your research interests, and the scientific tools you use to pursue them, seem to cover a broad spectrum. How would you characterize the work you typically do in your own labs, in relation to the collaborative interplay fostered by the TUM-IAS Focus Group?

TM: You could say we're trying to do ambitious things across long distances – biologically, between yeast and mice, and geographically as well.

MS: In my lab here in Israel, we try to understand how cells, the basic units that build up our bodies, are organized. All of our cells are compartmentalized into functional areas called organelles, which basically are membrane-enclosed compartments where specific biochemical reactions can take place. And they enable the cell to diversify the number and types of reactions or functions that can occur in parallel. This is an important aspect of how our cells are built, and I would like to understand how these organelles are organized in the three-dimensional space of the cell and how they communicate with each other. Because if you have these entities, and each performs several functions, in the end you also need to coordinate their functions with one another and make sure that it's all supporting this one big cellular community. We do this in a very simple cell. It's a model cell of the fungus called Saccharomyces cerevisiae, better known as baker's yeast or brewer's yeast. The wonderful thing about it is that the basic cellular functions of this fungus – even though we diverged in evolution about a billion years ago – are very similar to our own cells.

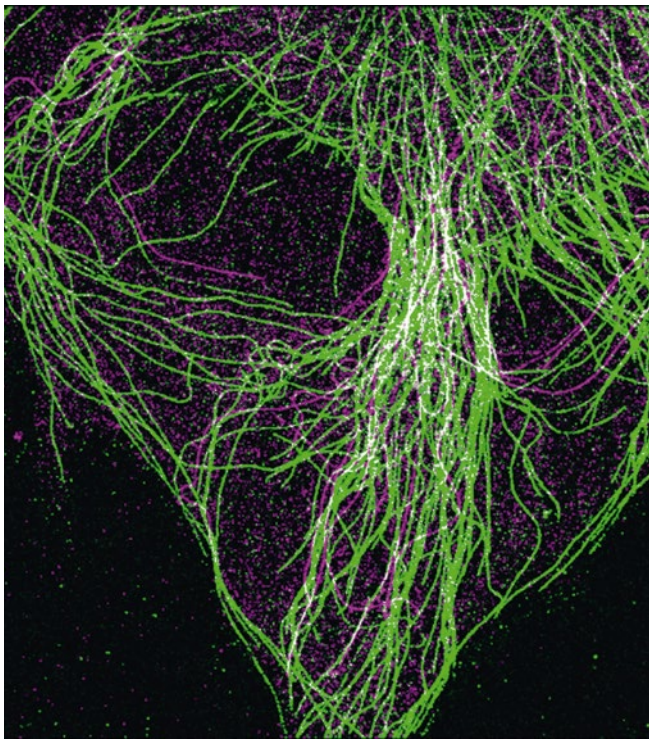


Nerve cell processes (green) with labeled mitochondria (blue) in the tail of a zebrafish larva.

»The wonderful thing about it is that the basic cellular functions of this fungus – even though we diverged in evolution about a billion years ago – are very similar to our own cells.«

The collaboration with Thomas allows us to explore how the principles that we find in yeast are conserved to mammals, such as mice and humans, and to try to understand how these principles are built on or diversified to sustain not only a basic cell but also the many different types of cells that we have in our bodies. On the one hand, we can look at the things that are similar between all cell types – because if it's similar in a neuron in a mouse brain, it means that every single eukaryotic cell will behave the same way. If this whole diversity still maintains the same basic functions, it's probably the same everywhere you look. And if we find cases or scenarios where unique differences between the yeast system and different mammalian cell types can be identified, this could help us recognize what types of functions have to be modified to support different types of functions, for example, in a brain.

TM: The neuron, in addition to being in a different organism and having a very different geometry, also has further levels of compartmentalization. There are some parts of the neuron where the organellar composition is what we conventionally associate with a cell in general, but there are other parts, like the axon and the synapse, that don't have the full complement. We're only starting to discover what is actually there, and given that everything has to be shuttled out there, it's actually quite interesting in which kinds of forms and interactions that happens, and whether the



Multi-color super-resolution image of tyrosinated (green) and detyrosinated (magenta) microtubules.

interactions between organelles are maintained during transport or whether the things are brought into the periphery of the cell separately and then re-formed in those contexts.

The starting point for doing these kinds of investigations is finding molecular definitions of these contacts. That's what Maya has spearheaded in the yeast, looking at pretty much any permutation of organelles you can think of and asking which molecules would mediate between them. These are typically pairs of molecules that tether together and create some kind of intracellular adhesion between organelles.

MS: For example, we discovered a couple of years ago that the mitochondrion, which is the organelle where we convert sugar to energy that can be utilized by the cell, and the peroxisome, which breaks down fats, also to provide an energetic source for the cell, are physically attached to one another through an area called a contact site. They literally contact each other, they are coupled, they move around the cell together. But it was not known what mediates this connectivity. So we devised a visual tool to look at only places where the mitochondria and the peroxisome are connected at this contact site. We coated all of the mitochondria with half a fluorescent protein, coated all of the peroxisomes with another half of the fluorescent protein, and only when they are in very close proximity, such as that which you find in contact sites, the two halves of the fluorescent protein come together and you see a signal. And now we could use this as a visual assay to look for proteins that affect this contact site.

So we screened genetically for proteins that affect the signal when overexpressed or deleted. And we found one protein – in yeast it's called Pex34 – that when you overexpress it, you can dramatically expand the amount of contact that these two organelles have. And we showed – with many different assays – that this Pex34 is really what holds the two organelles together and enables the transfer of the lipid breakdown product into the mitochondrion for conversion to ATP. Now it turns out that this Pex34 is conserved all the way to humans. Its human homologue is involved in a lot of very severe peroxisomal diseases, where the children who are born with mutations in this homologue have very bad neurological disorders, and they die at an early age. The question is: Is the same function that we found in yeast also conserved all the way to mammals, or to humans? So this is a place where we can really start to look. We've just finished making the same probes for a mouse cell, in collaboration with an Italian lab headed by Dr. Tito Cali.

TM: And so the starting point is to take what we know from yeast and try to find mammalian homologues and ask whether these mammalian homologues are in the nerve cells, and then to ask in which part of the nerve cell they are present, and ideally find ways to visualize them three-dimensionally. That is a forte of my lab, and here we can use the mouse as the model organism or use the zebrafish as an intermediate step. Some organelle contacts are fairly well characterized, like the ones between mitochondria and the endoplasmic reticulum. Both of these organelles not only play important roles in biosynthetic steps but also interact, for example, in terms of calcium-handling interactions and in lipid metabolism. It's very clear that these contacts, between mitochondria and the

Top research: Thomas Misgeld and Melike Lakadamyali in early discussions on the Wank summit near Garmisch Partenkirchen, 2009.

endoplasmic reticulum are disrupted in a lot of neurological disease settings like Alzheimer's. But there are many other organelle combinations that are unstudied.

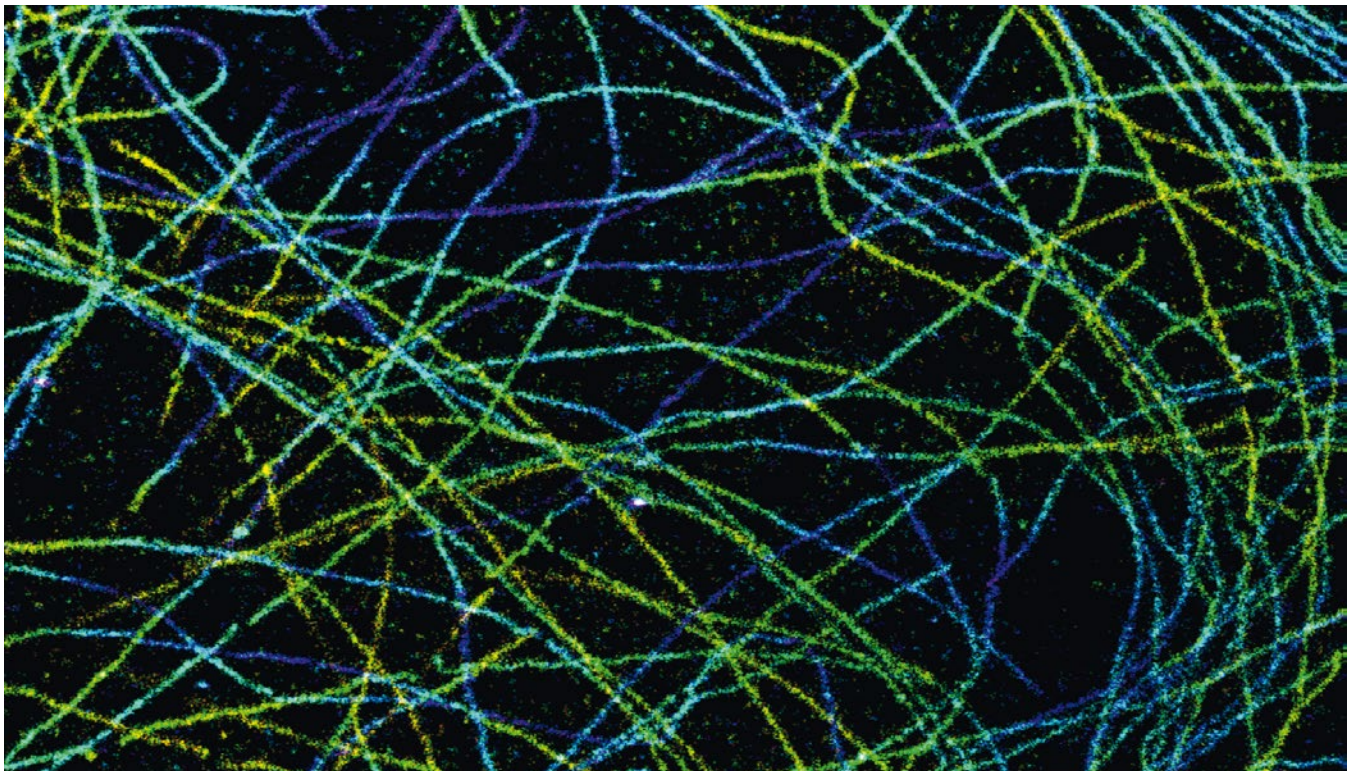
When you come from yeast to the brain, it gets a bit more complex. One way that we are approaching this is by using antibodies for this specific protein, the homologue of Pex34 called Pex11beta/gamma, to analyze mitochondria from neurons and from the neighboring cells, the glial cells. A defect in either can give you a brain disorder. To achieve this, we coated the surfaces of mitochondria with a full fluorescent protein, but in a way that allows us to decide, by breeding the animals, in which cell type that would happen, glial cells or neurons. Because there are very good antibodies for GFP, green fluorescent protein, you can not only see the mitochondria, but you can also grind up the brain and – with little iron beads and a big magnet – pull out only the mitochondria that came from either neurons or glial cells. Then you can ask: What is hanging onto my little iron beads? If something is strongly tethered to the mitochondria in this cell, that other thing should also be there. So you can ask: Do we ever pull out something that a mass spectrometer will tell us is, say, peroxisome instead of mitochondria? This happened – not in neurons, though, but in glial cells. This suggests that peroxisomes are attached to mitochondria very strongly in glial cells.

In the end you would want to target this protein in a way that is cell-type specific and ask: Will that change something about that relationship, and will it have functional consequences? The question might be that if you get this phenotype not in neurons but in glial cells, would that be compatible with the clinical picture of patients? Knowing the answer could play a role in how you make a model of a disease in an animal and how you target the underlying effect clinically. That shows how this combination of tools allows us to follow up on things we actually didn't anticipate.

Q: Melike, in addition to being based several hours west of Thomas and Maya, you're connecting to this from a different direction technically, aren't you?

ML: I'm especially interested in intracellular transport. Intracellular trafficking along the microtubule cytoskeleton is an important biological process in all cell types, but particularly in neurons, because they have very long structural projections and depend on long-range transport to get materials to the right place. We know the individual components that are involved in intracellular trafficking. We know proteins have to attach themselves to cargoes, subcellular compartments, and walk along microtubules to transport these materials from one place to another. But how these work in the complex environment of a living cell in the presence of obstacles – "roadblocks," if you will – is not clear because so far this has mostly been studied by reconstitution *in vitro*, that is on glass slides, where really high-resolution imaging tools can be used to probe the process. These *in vitro* reconstitution experiments are in an unrealistically simplistic environment that doesn't take into account the full complexity of the cellular environment. So the aim of my group is to bring these tools into the cell context and try to probe this process inside the cell at an unprecedented level of detail.

In my lab, we use and further develop microscopy and imaging tools, including super-resolution microscopy, that allow us to visualize processes inside a cell. Tools like two-photon microscopy or confocal microscopy are typically used to visualize cellular structures and compartments and molecular machinery by labeling them. These have taught us a lot, and they're great tools, but they're not so well suited to imaging the machinery that is involved in transport on the microtubule tracks. A microtubule is a long structure but very skinny, about 30 nanometers in diameter.



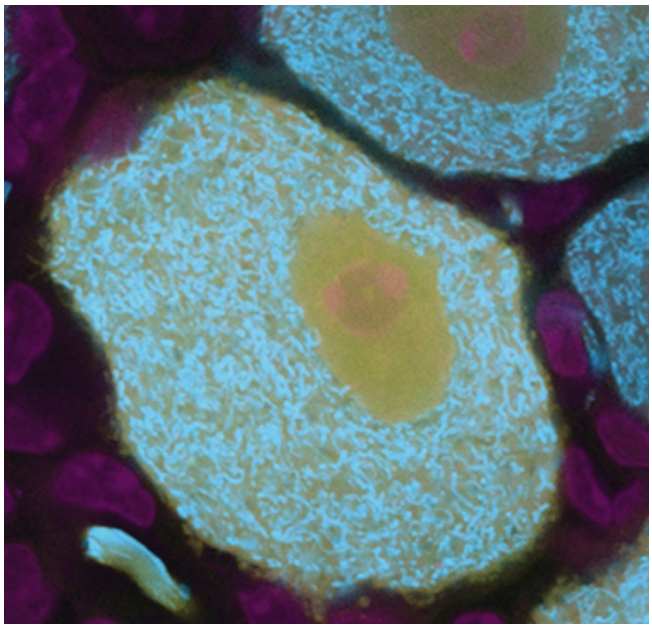
3D super-resolution image of microtubules color-coded according to depth.

The motors that work along these microtubules are on a similar scale, 20 to 30 nanometers. With tools like two-photon and confocal microscopy, we cannot visualize structures that are smaller than about 300 nanometers. That is the limit imposed by the wavelength of light. The tools that we're using and further developing break the limitation imposed by the wavelength of light and give us spatial resolution that is much higher. With this so-called super-resolution microscopy – for which the 2014 Nobel Prize in Chemistry was awarded – we can really visualize the complexity of the cellular cytoskeletons, the microtubule architecture, and where the roadblocks are at a great level of detail and try to understand how motors work in that complex environment.

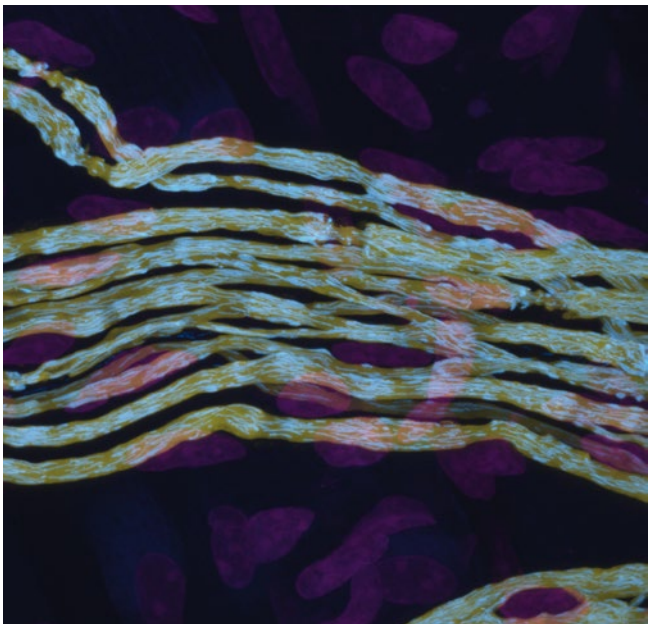
From the technical side, I think this ability to go below the diffraction limit, or the resolution limit of light microscopy, really was quite an exciting development. And we have been slowly pushing that limit to smaller and smaller scales. We're not quite at the molecular scale yet, but I think with new developments we will get to a level where we can look at individual molecules, individual complexes, inside a cell. That will be really exciting.

»I think with new developments we will get to a level where we can look at individual molecules, individual complexes, inside a cell.«

TM: I think it's important here to stress that in contrast to me and also Maya, Melike is a physicist. These super-resolution methods, either on the optical level or in the modeling that you have to use to actually extract information, are very sophisticated and well beyond my capabilities. I think one thing that is challenging – and very easily underestimated because we're so used to snapping a picture with a camera – is to be quantitative about those things on that scale. That's where the real power of this lies, because when you are now looking at small molecular assemblies, suddenly counting things becomes really important but far, far from trivial. People like Melike are really pushing that, and that's something people with my background will never be able to do.



Mitochondria (cyan) in sensory neurons (yellow) of a mouse - nuclei are labeled magenta.



Neuronal mitochondria in nerve cell processes (yellow) that innervate muscles of a mouse - nuclei of surrounding support cells and muscle are labeled magenta with a DNA dye.

ML: We've really been trying to go beyond, as Thomas says, snapping a pretty picture. To do that we are trying to understand, on a quantitative level, what the picture is telling us, and that is really challenging with these techniques. We've been developing methods to try to overcome those challenges as well.

Q: Are there more examples that show how the different lines of research are already reinforcing each other or coming together to achieve something new?

ML: We are normally looking mainly at epithelial cells, which are much simpler than neurons, but otherwise there is quite a lot of overlap. Lately we have been trying to understand the interplay between the motors and the cytoskeleton in the context of a biological process called autophagy, which means self-eating. It degrades cellular proteins to create amino acids to make new proteins, and it's also a process to help clear unwanted protein aggregates and damaged organelles like mitochondria.

For autophagy to happen, two compartments have to find each other inside the cell: what we call a lysosome, which contains all the enzymes that do the degradation, and an autophagosome that contains the unwanted proteins that damage organelles.

They have to meet each other, and they have to fuse, and these proteins are compartmentalized so that chemical reactions happen in a spatially and temporally controlled way. We have been wondering how these two compartments – in this really complex environment, where you have microtubules everywhere connecting every part of the cell and these organelles, if you look at them in a microscope, that seem to be just shuttling back and forth in the cell – find each other efficiently when they need to and fuse with each other. And we have been studying something called post-translational modifications of microtubules. Not every microtubule track is the same, and it is thought that chemical modifications to the microtubules may carry a code that the motors interpret.

Part of what we showed in our latest work is that yes, indeed, one type of modification is very important for enabling the encounter of these two compartments. There's a modification that is found in a very small population of microtubules – only about 30 percent of microtubules in the cell have this modification – and these two compartments that need to fuse with each other are enriched on these microtubules. And because there are only a few of these tracks, and these compartments travel largely on these few tracks, they can meet each other much more efficiently and fuse

with each other to initiate authophagy. Together with Thomas, we are looking at a specific form of autophagy, mitophagy, which is clearance of damaged mitochondria in the cell. We have looked at this in our lab in non-polarized cells, and now with Thomas we are looking at it in polarized cells like neurons, which are structurally very compartmentalized.

Q: Have you seen anything yet that surprised you?

ML: Yes!

TM: What came out of that study is that even within a subcompartment of a neuron, specifically the synapse – we're talking about neurons that have very large synapses, on muscles – there seems to be a geometric organization of this formation of the autophagosome and the lysosome. The lysosome seems to sit in a very specific position at the entry of the synapse. We don't know exactly at which step of autophagy yet, but at some point when mitochondria are fated to be removed, they move to this entry point. And rather than entering the axon and going all the way back to the soma – which is one of the assumptions about what they might be doing, and they might in fact be doing that in *in vitro* settings – here they seem to fuse locally and be degraded, meaning that there probably is a localized system of degradation.

Q: Would you call this a recycling center?

ML: More like a filter.

TM: Yes, a filter. But it also means that there is a local degradation. For the longest time, we have been puzzling why more mitochondria are shuttling into synapses than are coming out. There was always a mass discrepancy. And now using various higher-resolution approaches combined with electron microscopy, we have been able to do a mass balancing. Because of course you could say, well, you're shipping in more but what's coming back is bigger. So you have to measure very precisely the size of these cargoes in these different places to do a mass accounting. And that's something we have actually been able to do together, also using photoactivation to label individual organelles – where you have fluorescent proteins and you can change the colors. That allows us to label

Hoping for a breakthrough: Thomas Misgeld and Maya Schuldiner near the Danube Gorge ("Donaudurchbruch") with the Weltenburg Abbey in the background, 2018.

individual organelles, and we can now fix them and go to Melike's lab and measure them very precisely. It turns out mitochondria are long enough to measure them well with our microscopes, but the thickness is just at the level of the light wavelength, and so to decide how fat the mitochondrion is, we have to go to Pennsylvania. From that point of view it actually fits very nicely together.

It's conceivable – though we haven't looked at it, we haven't proven it – that there are actually very specialized microtubule modifications in the synapse itself, with some forms being relatively sparse over others, and we're very interested in the cytoskeleton as well. It's possible and could even be that the lysosome sits at the convergence point of these tracks. We haven't explored that, and perhaps we should.

ML: We need to look at that. I think this idea that compartmentalized autophagy can happen within the compartments of a neuron is really exciting. And it could be that different types of cargoes get degraded via autophagy that happens in these different compartments. And then, we know that mutations in a lot of neurodegenerative diseases impact proteins associated with autophagy. It would be interesting to probe whether they impact autophagy in general or they impact autophagy that happens within a specific subcompartment of a neuron. So these are some of the questions for future study.

TM: You could argue that this Focus Group has the same topic as the TUM-IAS overall: long-distance transport to create lasting contact sites. That's what we're doing in the Focus Group.

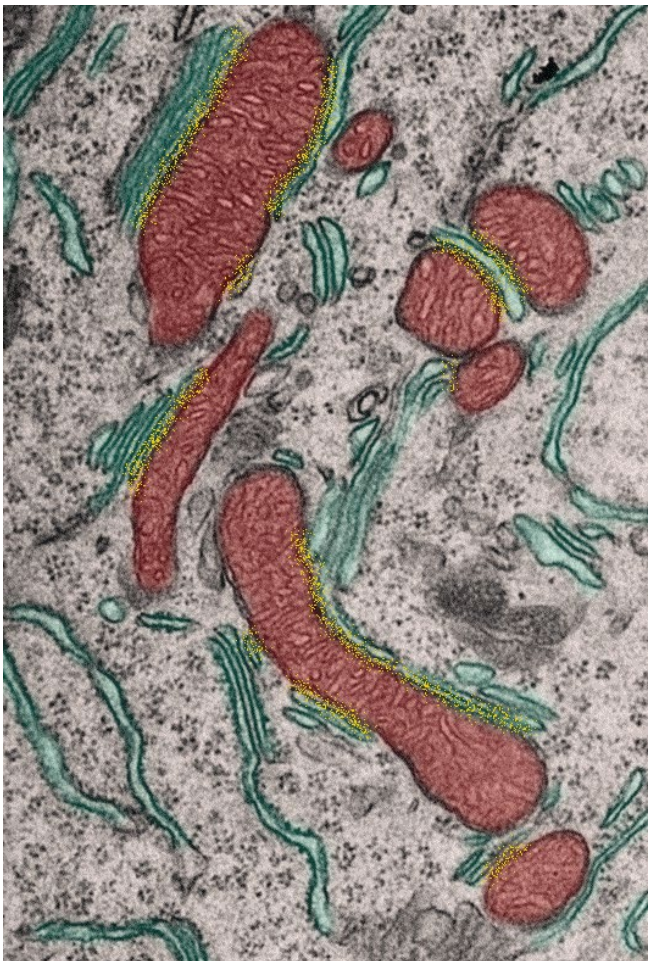
Q: How has the special framework of TUM-IAS Fellowships supported and shaped your collaboration?

TM: Fortunately for us, the TUM-IAS framework has shown a lot of flexibility, particularly with respect to Melike's Fellowship. TUM-IAS Fellows are expected to spend a significant amount of time here in Munich, for many good reasons. At the time we were arranging this, Melike was living in Spain, and her husband was free to go on sabbatical. But first Melike found out that she was expecting a child, and then she was offered a professorship at the University of Pennsylvania. So now she moved to America with a newborn, had to start up her lab in a new place, and was now in a situation where her husband would not be able to take a sabbatical in Munich. We proposed, and the TUM-IAS agreed, that Melike would come here for a few short visits instead of an extended stay. She has been here a couple of times for a week or ten days, and my people go there, to the University of Pennsylvania. This is great in terms of how the collaboration works, even though it is not optimal for the TUM-IAS. We are working very closely together on a project that is approaching publication.

Maya, on the other hand, was able to spend a full year here in Munich, with her whole family. Her husband Oren Schuldiner, who also is a highly distinguished scientist, was awarded the prestigious Bessel Research Award of the Alexander von Humboldt Foundation, which enabled him to come to Munich. And their three kids were able to attend the Bavarian International School.

Q: How did that work out for you and your family, Maya?

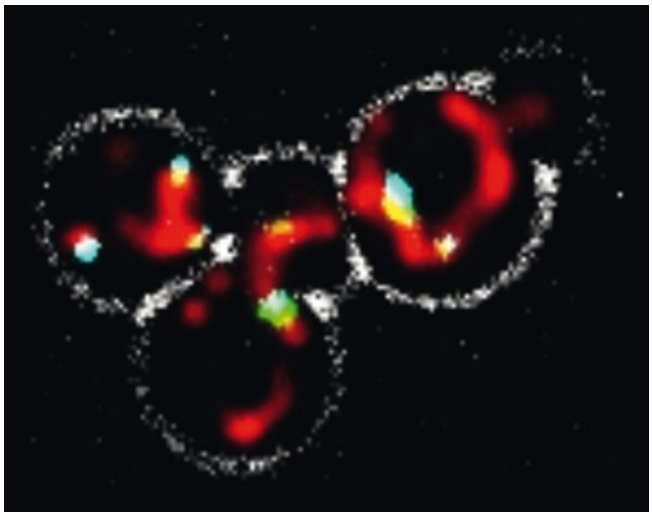
MS: This was really one of the most amazing experiences in my scientific career. I was able to work in Thomas's lab and join group meetings and take part in some TUM-IAS meetings, and really experience, first of all, how it is to work in a lab that has a very different expertise from mine. Also, this was a great opportunity to work in a city and a country that are very different from Israel, and to experience the cell biology in Germany, which is outstanding. It's really the best in the world in my eyes.



Close contacts between mitochondria (red) and the endoplasmic reticulum (cyan) are artificially rendered in yellow. The image shows an electron micrograph from Purkinje cells in the mouse cerebellum.

»You could argue that this Focus Group has the same topic as the TUM-IAS overall: long-distance transport to create lasting contact sites.«

I spent a year mostly in Thomas's lab, but I also took time to visit many other labs and many other cell biology departments all around Germany. I probably visited 20 different universities during this time. It was an amazing scientific experience.



Microscope image of a yeast cell showing how close mitochondria (red) are to peroxisomes (blue) and the contact sites that hold them together (green).

One of the things that people might not appreciate: In Israel, we have five universities, and if we want to go to any other university, we have to get on a plane and fly for a minimum of three and a half hours until we get to Europe. We're surrounded by countries that are either hostile, and we can't go there, or by countries where science is less advanced. When I was sitting in Munich, I would hop on the train at 7 a.m. and by 10 I could be anywhere – in Austria, Switzerland, Germany, France, Holland, the Czech Republic. Suddenly there are tens of universities that are just a couple of hours away by train. I felt like a kid in a candy store. Where do you go to first, when you can just hop on the train and get there?

The intellectual opportunities are amazing. And besides that, we loved living in Munich, and we were almost neighbors of Thomas's, just one street over. Our children were fifteen, twelve, and five at the time we arrived in August 2017. We stayed until August 2018. All the kids fell in love with Munich. My eldest son already told us that he's coming back to Germany to do his college studies.

TM: For us it was quite difficult and emotional to have them leave. That was one of the best things the people in my lab have experienced. We had training sessions with Maya for people who went for ERC interviews, and I think all of my people had one-on-one meetings with Maya and Oren for career advice. On their own campus, they are very engaged in outreach, and with gender and diversity issues. She's a very inspiring role model, a successful scientist who is a mother of three, with a husband who has been there for the children as much as she has. Scientifically, Maya and Oren had strong links to Germany before, but I think their year in Munich has really fortified them. They used the time to really get to know the science environment here.

Q: How is the Focus Group collaboration working for its earlier-career members, the doctoral candidates and postdoctoral researcher supported by the TUM-IAS?

TM: Our postdoc, Shabab bin Hannan, has moved on. He took a job at Tübingen. We have two doctoral candidates. Natalia Marahori, who is working on the project with Melike, is actually a medical doctor. She came to me in a specific program that we have in the TUM School of Medicine, called Translational Medicine, targeted at medical students who want to do a bit more science. They can do this for a year. And then, inspired I hope by interactions she had through that, she's now full-time. She's finished her medical degree and is here with us for a period of three years. She left the Translational Medicine program to upgrade to a full PhD. She's been in Philadelphia a couple of times already, imaging there. She's an absolutely outstanding person, has been with us for the whole length of the Fellowship, and will be the lead author of the study we are wrapping up.

Antoneta Gavoci, the doctoral candidate I am sharing with Maya, came to us from Italy. She's trained in molecular medicine and biological disciplines. She's been here for a year working on two tracks. One is to do what we originally proposed, trying to see whether these methods from yeast can be moved into our system. That proves to be a little more tricky. We may have underestimated the long-term effects of these modified tethers that we use as imaging tools. So what Antoneta is working on in parallel is to create an inventory of the cells at the molecular level, so that you can simply look at what, of the things that Maya finds in yeast, we also have in the specific motor neuron cells that we study.

And the projects are now also intersecting, because we decided to look at a mutation in a specific protein called spastin. That is mutated in patients with a disease called hereditary spastic paraplegia, essentially a motor neuron disease. The interesting thing about that protein is that its job is to cut microtubules. So it regulates transport, and we're now back to the idea that there are specific tracks inside of neurons that cargoes use. It turns out that spastin doesn't indiscriminately cut microtubules; it does it specifically in response to the modifications that Melike spoke about. It might be one of the proteins that read the code. So we decided that if Antoneta is doing an inventory, she might as well do an inventory not only of the normal situation, but also of the situation where that protein is missing, in order to understand how the cell responds to the challenge of having this code reader disrupted.

Q: When you're investigating something that can cause or at least influence a disease when it goes wrong, aren't you also learning more about how biology works when things go right? To what extent are these studies aimed toward understanding and curing diseases, and to what extent are they aimed toward better fundamental understanding of nature?

MS: I don't think you can differentiate between the two. Say you want a mechanic to fix your car. He can't fix your car unless he learns how every piece is supposed to be working when the car is functional, and then where things can go wrong. You can't fix something unless you know how it's supposed to be, and what the opportunities are when it goes wrong. The cell is a machine. It's a very complicated machine. It has a couple of thousands of parts. But it's finite, just like a washing machine or a car, and those parts have to be in a specific place and play a specific role. And if they're not sitting in the right place and doing the right thing, then the machine goes wrong, and you might be able to fix it if you know enough about it.

I do think some people primarily study disease states to try and understand what goes wrong. I think that it's much more powerful to look at what happens in a native state, because when you understand what goes right, it immediately becomes clear what can go wrong. This is why I study cell biology and not cancer or another specific disease. When you understand the principles of how things work, you immediately understand how they don't work.

TM: I would go a step farther. *Ex officio*, I should be saying we do it for disease. But I would differentiate between cells and washing machines and cars in that if nothing ever went wrong with washing machines or cars, I don't think anyone would study them. We would just accept that they're there and they're fine. This would not be true for cells, or the universe. We would study them even if they didn't go wrong. It's bad that they go wrong, and it's great that we can potentially fix them. But I would be happy to study mitochondria and peroxisome interactions, or mitochondria alone, just because it's deeply fascinating – how a nerve or even a yeast cell can simply work, just that it is possible, that this exists in the universe. I agree that for disease we need to understand it, and that's a useful side-effect of fundamental discoveries. But the simple fact that there is a physical chemical mechanism that can create something like a cell, which manages to survive and does so in such a sophisticated way – it's just marvelous that that can even work.

Right: Multi-color super-resolution image of detyrosinated microtubules (cyan) and lysosomes (yellow).