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Cover story

CONTENTS

3 Editor’s letter
4 News
10 Profile Jan Huisken
13 Caliciviruses: A tunnel to initiate infection Michaela J Conley
16 Fast volume imaging with bessel beam tomography and digital video processing by Andres Flores Valle and Johannes D. Seelig
18 Lab focus Peng Chen
20 4D-STEM with a direct electron detector Barnaby D.A. Levin, Chenyu Zhang, Benjamin Bammes, Paul M. Voyles, Robert B. Bilhorn
23 Meeting report
26 Product focus Digital cameras
26 What’s new
30 Company spotlight Protochips

EDITOR’S LETTER

DEAR FRIENDS, Welcome to our first issue of 2020! I hope you’re getting stuck into the New Year and the new decade! I can hardly believe it’s been five years since I became editor of M&A! I’m excited to see where our field will be in another five years.

This issue we’ve our usual diverse line-up of scientific editorials covering areas including 4D STEM, Bessel Beam tomography and Cryo-EM of virus structures. There is one inescapable requirement to all our modern microscopes, the image acquisition systems, and this issue our product focus is digital cameras. The spotlight focuses on John Damiano from Protochips detailing the company’s journey in the growth area of in situ microscopy. We also have a fascinating profile with a pioneer of light sheet microscopy Jan Huisken. He walks us though his beginnings in physics, the development of the light sheet through to his latest work, Flamingo. We also visit the lab of Peng Chen in Cornell as he and colleagues develop COMPETS, a super-resolution microscopy technique especially for non-fluorescent samples. Don’t miss the news and What’s New section to catch up on commercial developments. I also report from the November’s Cryo Microscopy Group (CMG) meeting. An issue with something for everyone!

CHRIS

EDITOR

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2020 vision

DEAR FRIENDS, Welcome to our first issue of 2020! I hope you’re getting stuck into the New Year and the new decade! I can hardly believe it’s been five years since I became editor of M&A! I’m excited to see where our field will be in another five years.

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CHRIS
US-based researchers have combined extreme speed photography with phase contrast microscopy to image ultrafast phenomena in transparent objects at picosecond resolution.

So-called phase-sensitive compressed ultrafast photography (pCUP) takes an incredible 1 trillion frames per second.

pCUP pioneer, Professor Lihong Wang, Caltech, has used the system to capture a shockwave created by a laser striking water and laser light travelling through a crystal, in a single shot.

Wang and colleagues combined phase-sensitive dark-field imaging with compressed ultrafast photography (CUP), based on the world’s fastest camera that they developed just over a year ago.

As Wang and colleagues explain in Science, CUP uses compressed sensing theory and the streak camera technology to achieve receive-only single-shot ultrafast imaging of up to 350 frames per event at 100 billion frames/s.

“Since CUP operates as a passive detector, it can be coupled to many optical imaging systems,” says Wang. “By combining CUP with dark-field microscopy, we show that pCUP can image ultrafast phase signals... at an improved speed of 1 trillion frames/s.”

Wang says the technology is still in its early stages of development but could ultimately have uses in many fields.

**Ultrafast camera takes 1tn frames per second**

**TEM captures bonding in molecules at the atomic scale**

Researchers from the National University of Singapore have synthesised the world’s first one-atom-thick amorphous material. Atomic resolution imaging revealed the complete absence of long-range periodicity in the monolayer amorphous carbon (MAC), which the researchers hope will settle decades of debate on how atoms are arranged in amorphous solids.

“With MAC, we have shown for the first time that fully amorphous materials can be stable and free-standing in single atomic layers,” says Professor Barbaros Özyilmaz, Head of the NUS Department of Materials Science and Engineering. “Amorphous materials are of great technological importance, but surprisingly, they remain poorly understood from a basic science point of view.”

Using laser-assisted chemical vapour deposition, Özyilmaz and colleagues deposited a stable monolayer of amorphous carbon onto a substrate. They then used Raman and X-ray spectroscopy, and transmission electron microscopy to characterise the film.

In past studies, researchers had suggested that these materials could either have a fully-disordered, completely random structure or display nanometre-sized order of unsupported Re-Re bonds using single-walled carbon nanotubes as a ‘nano-test tube’ while simultaneously imaging the structure and dynamics of the single atoms in real time.

“Nanotubes help us to catch atoms or molecules, and to position them exactly where we want,” explains Khlobystov. “In this case we trapped a pair of rhenium atoms bonded together to form Re. Because rhenium has a high atomic number it is easier to see in TEM than lighter elements, allowing us to identify each metal atom as a dark dot.”

Research is published in Science Advances.
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Grown in the lab: miniature brain tumor with blood vessels

UK-based Cambridge University researcher, Agavi Stavropoulou-Tatla, has unveiled a stunning image of a mini brain tumor with blood vessels, grown in the lab. So-called tumors are miniature versions of tumors grown in laboratories to offer a robust platform for the development of personalised therapies. Stavropoulou-Tatla is currently studying for her PhD, and as she says: "This biomimetic model could provide insight into the mechanisms that drive tumor new blood vessel formation and invasion along blood vessels, and serve as a personalised tool for targeted drug testing."

Stavropoulou-Tatla captured the image using epi-fluorescence microscopy; a Zeiss Axio Observer Z1 inverted microscope with an ORCA-Flash4.0 camera and a 10x lens. The image is the result of the deconvolution of an image z-stack. The image shows a growing tumor that can be used to study the interaction between brain tumor (glioblastoma) cells and blood vessel forming (endothelial) cells. Both types of cell types have been genetically modified to express green and red fluorescent protein respectively.

According to Stavropoulou-Tatla, this type of brain tumor, glioblastoma, is able to grow so quickly because it has the power to produce new blood vessels when needed. "It is highly infiltrative, and this is partially because tumor cells use blood vessels as 'highways' for their migration to different parts of the brain," she says. Stavropoulou-Tatla's initial image won the microscopy prize in the University of Cambridge, Department of Engineering ZEISS Photography Competition.

How microscopy has influenced literary legends

UK-based electron microscopist, Dr Peter Harris, University of Reading, has recently published an article entitled Microscopy and literature, which takes a look at how microscopy has influenced literary works.

Publishing in Endeavour, Harris shows, for example, that the publication of Robert Hooker’s Micrographia (1665) influenced Jonathan Swift’s book Gulliver’s Travels (1726).

As he points out, in the chapter ‘A Voyage to Brobdingnag’, Gulliver becomes a kind of ‘human microscope’, observing with his naked eye the spots, pimples and freckles on the skins of the giant Brobdingnagians in minute detail.

Other examples of microscopy in literature are taken from works by Voltaire, George Eliot, H G Wells and D H Lawrence.

The inspiration for the article came from a conversation that Harris had with Professor Steven Matthews from the English Department at Reading. While explaining that his job involves running the University’s electron microscopy facility, Matthews had said that the only reference to microscopes in literature that he could think of was in Lawrence’s The Rainbow, where the heroine, Ursula Brangwen, experiences a revelation about the meaning of life while peering down a microscope.

"This set me wondering whether there were any other examples of microscopy in literature," says Harris. "It turns out that there are many more than you might think."

Deep learning restores under-sampled images

Using deep learning, US-based researchers have developed a method to accelerate neuroimaging by up to sixteen times.

The new imaging method, called point-scanning super-resolution (PSSR) imaging, can restore under-sampled SEM and laser scanning confocal microscopy images, overcoming the resolution, speed, and sensitivity trade-offs associated with point-scanning image acquisition.

"Not only does this approach work, but our training model can be used right away," says Uri Manor, Director of the Waitt Advanced Biophotonics Core Facility at the Salk Institute for Biological Studies in San Diego. "It's extremely fast and easy, and anyone who wants to use this tool will soon be able to log onto 3DEM.org, the web-based research platform for 3D electron microscopy, and run their data through it," he adds.

While scanning electron microscopy and laser scanning confocal microscopy are widely used in high resolution cellular and tissue imaging, resolution, speed, sample preservation, and signal-to-noise ratio are difficult to optimise simultaneously. With this in mind, Manor, Linjing Fang also from Salk, and colleagues, developed a deep learning-based super-sampling system that can be applied to under-sampled images, which they call point-scanning super-resolution (PSSR) imaging.

Manor first obtained images from Professor Kristen Harris from the Department of Neuroscience at the University of Texas Austin that had been acquired on both scanning electron and Aryscan laser scanning confocal microscopes.

Then, working with Jeremy Howard, founder of fast.ai, and Fred Monroe, from the Wicklow AI Medical Research Initiative, they ‘crappified’ the oversampled, high signal-to-noise ratio images to generate low-resolution analogs, with the image pairs then being used as training data for PSSR models.

These models were then used to restore real-world under-sampled images, creating high-resolution images that were very similar to the ones that had been originally created with greater magnification.

Moreover, researchers were also able to find brain cell features in ‘de-crappified’ versions of the low-resolution images that couldn’t even be detected in the original data.

"Remarkably, our electron microscopy PSSR model could restore under-sampled images acquired with different optics, detectors, samples, or sample preparation methods in other labs," highlights Manor in bioRxiv. "[The method] enabled previously unattainable 2 nm resolution images with our serial block face scanning electron microscope system."

The PSSR model could also convert under-sampled confocal images to Aryscan-equivalent spatial resolution and signal-to-noise ratio with around 100x lower laser dose and 16x higher frame rates than the corresponding high-resolution images.

"To image the entire brain at full resolution could take over a hundred years," says Manor. "With a 16 times increase in throughput, it perhaps becomes 10 years, which is much more practical."
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SEM unravels how hearing develops

SEM and fluorescence microscopy and have shed new light onto the development of hair bundles, the intricately complex assemblies in the inner ear responsible for hearing.

New results from Dr Jocelyn Krey, at the Oregon Hearing Research Center and the Vollum Institute at Oregon Health & Science University, US, and colleagues, reveal that hearing develops in tandem with form and function. The latest studies point to how scientists might develop techniques to regenerate hair cells and reverse hearing loss.

Hair bundles are precisely arranged cellular structures deep within the spiral cavity of the inner ear. Together, the structures convert vibrational energy into electrical signals in the brain that translate into the sensation of hearing. Once lost – whether by loud noise, toxins, disease or ageing - the bundles do not naturally regenerate in people and other mammals.

However, investigations on mice, by Krey and colleagues, revealed that the development of hair bundles occurs in a feedback loop, in which form follows function and function drives form. Image-scanning microscopy with Airyscan detection and SEM allowed the researchers to examine a stereocilia in a hair bundle.

Analyses showed that stereocilia widen simultaneously with the onset of mechanotransduction, the action of converting mechanical signals in the form of sound into electrical signals measured within the brain.

The stereocilia only elongated to their mature lengths after transduction had been established, indicating that form and function are mutually reinforcing.

“We’ve been looking at these as separate pathways,” says Krey. “But in the course of this research, we observed the change in form occurs at the same time as the conversion of mechanical to electrical signals. So we’re seeing these happen together, and feeding each other in a way we hadn’t seen before.”

The researchers now believe that new techniques to reverse hearing loss should focus on the critical importance of early development.

Research is published in Current Biology.

Steel embrittlement mystery solved

Australia-based researchers have discovered new evidence of how hydrogen embrittlement in high-strength steel takes place, and importantly, how to prevent it. While hydrogen atoms tend to diffuse and accumulate at dislocations and grain boundaries in a steel, cryogenic atom probe microscopy has shown exactly how niobium carbide clusters within its microstructure help to trap the hydrogen, reduce embrittlement and prevent steel fracture.

The latest results point to the design of embrittlement-resistant steels for stronger steel tanks and pipelines in hydrogen environments.

Hydrogen embrittlement of high-strength steel is a well-known obstacle for using these steels in sustainable energy production. While the phenomenon is known to involve the interaction of hydrogen and defects, issues around precisely locating hydrogen atoms throughout the steel microstructure have hindered investigations.

As Dr Yi-Sheng Chen from the Australian Centre for Microscopy and Microanalysis at the University of Sydney writes in Science: “The combination of the low level of interaction between an electron and hydrogen, and the extremely fast diffusion of hydrogen in steels renders it extremely difficult to experimentally determine the location of hydrogen by electron microscopy.”

With this in mind, Chen and colleagues, turned to cryo-transfer atom probe tomography to pinpoint the location of hydrogen at microstructural features. Results showed hydrogen pinned at incoherent interfaces between niobium carbides and the surrounding steel, providing direct evidence that these boundaries can act as trapping sites.

“These findings are vital for designing embrittlement-resistant steel,” says Chen.

Surprising beauty found in bacterial cultures

Using fluorescence imaging, researchers at University of California San Diego, US, have discovered that when microbes pair up, stunning floral patterns can emerge.

When Lev Tsimring from the BioCircuits Institute at UCSD, and colleagues, combined non-motile *Escherichia coli* with motile *Acinetobacter baylyi* on an agar surface, they noticed that the *E. coli* “catch a wave” at the front of the growing *A. baylyi* colony, with unexpected results.

“We were actually mixing these two bacterial species for another project, but one morning I found a mysterious flower-like pattern in a petri dish where a day earlier I placed a droplet of the mixture,” explains Dr Liyang Xiong from Physics at UC San Diego.

“The beauty of the pattern struck me, and I began to wonder how bacterial cells could interact with each other to become artists.” According to the researchers, while *E. coli* can’t easily move across agar, *A. baylyi* can crawl using pili; combine the two and intricate flower-like patterns form over a 24 hour period.

Using an inverted, epifluorescence Nikon Ti2 microscope with a Photometrics Cool-Snap cooled CCD camera, the researchers were able to image the surprising floral formations.

To unravel how these patterns form, Xiong and colleagues developed mathematical models to account for the different physical properties of the two bacteria strains, including differences in growth rate, motility and effective friction against the agar surface.

Theoretical and computational analysis showed that the pattern formation originates at the expanding boundary of the colony, which becomes unstable due to drag exerted by the *E. coli* that accumulate there.

Where there is less *E. coli* accumulation, there is less friction, allowing the boundaries to push out faster. And in regions with more *E. coli* accumulation and friction, the boundaries stagnate, creating the ‘petals’ of the flower pattern. The results have important implications in studying growing biofilms.

Research is published in *eLife*.
Researchers from The Netherlands and Germany have merged two super-resolution microscopy techniques to create a new method to image components within living cells with more precision than ever before.

By doubling the imaging precision compared to standard localisation microscopy, so-called SIMFLUX paves the way to new insights in healthcare and more.

While single-molecule localisation microscopy circumvents the diffraction limit, improvement over state-of-the-art image resolutions of around 20 nm towards 5 nm is necessary to truly image at the molecular scale.

With this in mind, Professor Sjoerd Stallinga from the Delft University of Technology and colleagues combined single molecule-localization microscopy with structured illumination microscopy in a single set-up, SIMFLUX.

Using the new method, they went onto image an artificially produced DNA structure, producing a clear picture of the proteins structures – between 5 to 10 nm in size – that form the cytoskeleton.

As the researchers write in Nature Methods: “We show a near two-fold improvement in precision over standard localization [microscopy] with the same photon count on DNA-origami nanostructures and tubulin in cells.”

The researchers believe the new method is a major step forward and now hope to image samples in 3D.

“The logical next step would be to make images in three dimensions,” says Bernd Rieger from the Department of Imaging Physics at Delft. “That’s another big challenge, but we already have some ideas.”

Research is published in Nature Methods.

### Ghost imaging speeds up super-resolution microscopy

China-based researchers have developed a method that uses ghost imaging to achieve super-resolution microscopy at unprecedented speeds.

Based on stochastic optical reconstruction microscopy (STORM), the new method is poised to capture the details of processes within living cells at higher speeds than ever before.

As the researchers highlight in Optica, the unconventional imaging approach produces nanometre resolution with far fewer imaging frames than traditional super-resolution methods.

“Our imaging method can potentially probe dynamics occurring on millisecond time-scales in subcellular structures with spatial resolution of tens of nanometres – the spatial and temporal resolution at which biological processes take place,” says Zhongyang Wang from the Shanghai Advanced Research Institute, Chinese Academy of Sciences.

Single-molecule, localization-based, wide-field nanoscopy suffers from low time resolution as localisation of a single molecule with high precision requires a low emitter density of fluorophores.

What’s more, reconstructing a super-resolution image demands hundreds or thousands of image frames, even when advanced algorithms, such as compressive sensing and deep learning, are applied.

With this in mind, Wang and colleagues developed a single-frame, wide-field nanoscopy system based on ghost imaging, in which a spatial random phase modulator is applied in a wide-field microscope to achieve random measurement of fluorescence signals.

The random phase modulator converts the fluorescence from a sample into a random speckle pattern.

Coding the fluorescence in this way allows each pixel of a CMOS camera to collect light intensity from the whole object in a single frame.

As the researchers write in Optica, the method enhances the imaging resolution to 80 nm, with the raw image being reconstructed using compressive sensing.

“While STORM requires a low density of fluorescent labels and many image frames, our approach can create a high-resolution image using very few frames and a high density of fluorophores,” says Shengsheng Han, Shanghai Institute of Optics and Fine Mechanics, Chinese Academy of Sciences.

Using the method, the researchers imaged a 60 nm ring, resolving the object using only ten frames.

In contrast, STORM approaches needed up to 4000 frames to achieve the same result.

The researchers now hope to speed up the method and achieve video-rate imaging with a large field of view, to acquire 3D and colour images.
When it comes to light sheet microscopy, biophysicist, Jan Huisken, has been there pretty much from the beginning. In 2004, while working on his PhD in the laboratory of confocal microscopy pioneer, Ernst H K Stelzer, he, Stelzer and colleagues shook the world of biology when they unveiled selective plane illumination microscopy.

While confocal microscopy had always relied on beam scanning to raster across a sample, SPIM drastically reduced the light directed at a sample by shining it in sheets, perpendicular to the detector. The end result was a gentle, high-speed method that could image animal and plant embryos for more than 100 hours, without damaging them.

“We’d been working hand-in-hand with biologists, and so realised how valuable this was for the community at the time,” says Huisken. “We were seeing a huge push from biologists towards imaging larger specimens, including tissues and whole embryos, at a time when many fluorescent proteins were also becoming available.”

“At EMBL you couldn't do anything without it eventually being beneficial to biology, and this is something that I embraced and has become a theme in my later laboratories,” he adds.

With SPIM developed, Huisken realised he wanted to take light sheet microscopy further afield and joined the laboratory of biologist and developmental geneticist, Professor Didier Stainier, at the University of California, San Francisco. According to Huisken, Stainier was running one of the largest zebrafish labs in the world at the time, but biology's favourite large, transparent organism wasn't so easy to image with traditional light microscopy.

“I was the only physicist in this lab, and my goal was to bring [SPIM] to a new research field,” he says. “Didier always said, ‘I don’t really understand very much of what you are doing, but I trust you’ and that was great – I had all this support and could try so many things that I wouldn’t have necessarily tried in a physics environment.”

Success ensued, and together, Huisken, Stainier and colleagues used the method to image the zebrafish heart and vascular development, unravelling how blood vessels and heart valves form and even using optogenetics to control the organism’s cardiac system.
“We were imaging the vascular system and heart to a level that nobody had ever seen before,” says Huisken. “We could see the beating heart of a living fish and that was really rewarding.” Importantly, Huisken also had his eyes opened to the impact that light sheet microscopy could have on the field of developmental biology. Well aware that future research and instrument development would demand a multi-disciplinary team of physicists and biologists, he applied for Group Leader at Max Planck Institute of Molecular Cell Biology and Genetics in Dresden, Germany. Come 2010, he was setting up his first laboratory. “The Institute had learnt about light sheet microscopy and realised the method was not going to be commercially available any time soon, so hired me to build these instruments and develop them further,” he says. SPIM development and zebrafish research began apace, and within a few years Huisken and his team had truly pushed back the boundaries of light sheet microscopy. They had successfully confined living zebrafish embryos in tubes for time-lapse imaging over several days, applied the first commercially available tunable lenses for fast, 3D light sheet imaging and then unveiled astonishingly high resolution views of the beating zebrafish heart, in 3D.

“We had a biologist working on sample preparation and imaging, and optics researchers building the microscopes,” says Huisken. “But we were also strongly focused on software development, so we could register movie stacks and reconstruct the heart in multiple colours and in 3D over time, which of course was an enormous effort on the data-processing side.” “I think this was a great example of how different disciplines can come together to make something happen that simply hadn’t been achievable at all with any other instrument,” he adds. Indeed, by now, the wonderful world of imaging with light sheet microscopy was gathering momentum. While in the past, biologists typically had to adapt their sample preparation according to the imaging technique, SPIM provided more freedom. “With our study on the beating zebrafish heart, things became more 3D and dynamic, and very inspirational for a lot of scientists,” points out Huisken. “Researchers were saying, ‘Oh yeah, I can look at a freely swimming fish or a worm crawling over a coverslip, or maybe study muscle activity in a fly’.”

Still, despite success, Huisken was only too aware of the cost and...
Meet Flamingo

Named Flamingo, as the original version of the microscope reminds Huisken and colleagues of the bird standing on one leg, the modular SPIM can be constructed in either the classic or inverted geometry for high throughput imaging of conventionally mounted samples. Importantly, it is designed around a central post – rather than being spread out across an optical table – and several instruments can be built for the same price as a single commercial system.

The microscope uses water-dipping optics for illumination and detection, and can image live samples such as fruit fly embryos, zebrafish embryos and larvae, organoids, spheroids and tissue sections. Light sheet uniformity is enhanced via rapid pivoting of the light sheet. And rapid volumetric multi-colour imaging can be achieved via a fast, low-noise camera, multiple laser lines and an emission filter wheel. Spatial resolution is given by the chosen lens and the pixel size of the camera and the system frame rate and number of planes per second is typically 40 to 400 (depending on the number of pixels). The instruments currently support samples up to some 5 to 6mm in thickness, and depending on sample transparency, tissues as deep as 500 µm can be imaged. (see: involv3d.org/flamingo)

Huisken and colleagues have done. The ‘Flamingo microscope’ has been designed as a suitcase-sized, portable SPIM that can be passed between labs. Once shipped to a lab, the instrument can be remotely configured by Huisken or a fellow researcher ready for use, and then shipped onto to the next project (see ‘Meet Flamingo’).

According to Huisken, prototype development is complete, and at the time of writing, three instruments had been built with two more underway. “We’ve shown the instrument at several conferences to see if it is just a crazy idea or if biologists actually want it, and the response has been overwhelming,” says Huisken. “It’s been amazing to see how many people are excited about the idea of getting a custom-built SPIM from us, and ultimately these instruments could be used in biological experiments, where commercial instruments don’t meet requirements.”

So what next for Huisken? Alongside colleagues, he now intends to develop new configurations of Flamingo in his lab. And using the same software across each system, he reckons they will be able to swiftly develop new modifications and add-ons for the instruments.

“We are aiming to democratise high-end light microscopy and bring it to labs for free,” he says. “I find this idea of iteratively improving the instruments with biologists very exciting.”

“I think that by going out and collaborating with biologists we are going to have much more of an impact than if we tweaked a single instrument that only exists in my own lab and isn’t accessible to that many researchers,” he adds.
Caliciviruses: A tunnel to initiate infection

DR MICHAELA J CONLEY
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INTRODUCTION
Viruses are intracellular parasites that depend on the host cell machinery to replicate and assemble progeny virus particles. In order to enter cells, viruses must bind to specific receptor proteins on the cell surface and trigger uptake via mechanisms such as endocytosis. Whilst enveloped viruses can enter cells by fusing their envelope with host cell membranes (either at the plasma membrane or in endosomes), the mechanisms by which non-enveloped viruses escape the endosome are poorly understood. Viruses must overcome this barrier to infection in order to deliver their genomes into the cytoplasm of host cells to then subvert the cellular machinery to their own end i.e. to replicate their genomes, produce their proteins and assemble progeny virus particles.

Caliciviruses are a family of viruses that infect many different species including cats, dogs, rabbits, sealions and humans. The most common calicivirus that infects humans is norovirus. Norovirus causes ‘winter vomiting disease’ resulting in diarrhoea and vomiting and is reasonably common throughout the world. Although norovirus infection is trivial to many in developed countries, it causes 70-200,000 childhood deaths every year, most of which are in developing countries[3]. Other caliciviruses also infect humans, such as sapovirus, and some even affect animals such as cats, dogs, rabbits, sealions and Fabrazyme, respectively) and Fabry disease (Cerezyme and Fabrazyme, respectively) which are found as a contaminant of bioreactors used to produce therapeutics for the treatment of Gaucher disease (Glybera) and Fabry disease (Cerezyme and Fabrazyme, respectively). Mammalian caliciviruses are also important veterinary pathogens in their own right. For example, feline calicivirus (FCV), is a widespread pathogen of domestic cats, found particularly in animal shelters and necessitating vaccination every one-three years[4]. Highly pathogenic strains of FCV have also emerged which are termed ‘virulent systemic’[5] as they cause a much more severe infection than the usual cold-like symptoms and oral ulceration seen with non-virulent isolates[5-8].

Until recently, norovirus was unable to be grown in the laboratory and so some animal caliciviruses have been used as a model to study calicivirus biology[11, 12]. FCV is one of the few caliciviruses for which a receptor protein has been identified and is arguably one of the most well characterised model systems for the study of caliciviruses, particularly one of the vaccine strains known as F9. The receptor for FCV is feline junctional adhesion molecule-A (fJAM-A) and is found at tight junctions between cells as well as on the surface of platelets and some cells of the blood[13, 14]. The fJAM-A ectodomain (the portion of the protein which is exposed on the cell surface) is the site of virus binding for both FCV and norovirus[15-17].

Calicivirus particles/capsids are 35-40nm in diameter and have t=3 icosahedral symmetry. The capsids are composed of 180 copies of the major capsid protein VP1. VP1 can be sub-divided into 2 domains: S (shell) domain which forms the contiguous shell of the capsid and the P domains which form the spikes which extend outwards from the capsid shell and include the binding site for fJAM-A and neutralising antibodies[16, 18-20]. VP1 capsomeres/dimers are present in three conformations which differ slightly to allow formation of an icosahedral shell (namely A, B and C). A/B capsomeres/dimers are found at the 5-fold symmetry axes and C/C dimers are found at the 2-fold symmetry axes, resulting in the alternation of A/B and C/C dimers around the 3-fold symmetry axes. Caliciviruses also encode a minor capsid protein, VP2, although until this study, the structure and function of the protein remained unknown despite VP2 having been shown to be essential for productive infection. We describe the study of FCV both unbound and decorated with the ectodomain of fJAM-A in terms of structure and the entry pathway utilised by the virus to initiate infection of host cells. We show that upon receptor engagement, the virus undergoes a conformational change allowing the extrusion of a portal-like assembly at a unique three-fold symmetry axis. We hypothesise that this ‘tunnel’ functions as a mechanism of genome delivery and endosomal escape to allow infection of the host cell to progress.

MATERIALS AND METHODS
Viruses were grown in Crandell reese Feline Kidney (CrFK) cells and purified using several stages of ultracentrifugation. Briefly, samples of infected cells were spun at 74,000 x g to pellet the virus, the virus was layered onto a caesium chloride gradient and spun at 134,000 x g, the virus was then extracted from the tube and pelleted a final time before use in structural experiments. The soluble ectodomain of fJAM-A was over-expressed in Chinese Hamster Ovary (CHO) cells and purified using an Fc tag via affinity chromatography.

For cryo-electron microscopy, virus alone or equal concentrations of virus and fJAM-A were mixed and added to a C-flat advanced holey carbon grid (ProtoChips) prior to plunging into liquid nitrogen cooled liquid ethane and stored under cryogenic conditions until inspection. Grids were imaged in a Titan Krios electron microscope (ThermoFisher Scientific) equipped with a Falcon III direct detection device at the Astbury Biostructure Laboratory (University of Leeds, UK). Micrographs were collected at a range of defocus values from 1.2 ± 3.5µm with a pixel size of 1.065Å per pixel. Micrographs were processed in Relion 3.1 using focussed classification and stored under cryogenic conditions to function as a mechanism of endosomal escape and genome delivery, allowing infection to advance.

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I am currently a Research Associate at the MRC-University of Glasgow Centre for Virus Research in the structural virology group of Professor David Bhella. My background is in virus entry (including structural, biophysical and molecular techniques). I received my PhD in structural virology from the University of Glasgow in 2018 as well as an MSc(Res) in virology and BSc (Hons) in Microbiology with Virology from the University of Leeds.

ABSTRACT
Caliciviruses include the notorious cause of winter vomiting disease, norovirus. Until recently, the mechanism of endosomal escape, required by some families of viruses to infect cells, was not understood for caliciviruses. Cryo-electron microscopy, three-dimensional image reconstruction and focussed classification were used to determine the structure of feline calicivirus alone and bound to its cellular receptor, fJAM-A. A novel portal-like tunnel assembly was shown to form at a unique three-fold symmetry axis in the viral capsid which is hypothesised to function as a mechanism of endosomal escape and genome delivery, allowing infection to advance.
refinement, post processing and local resolution estimation\cite{21}. Maps were visualised in UCSF Chimera\cite{22}. Focussed classification was used to further analyse the data set whilst not imposing any symmetry (previously icosahedral) in Relion\cite{11, 23-25}. Model building was performed using Coot and Phenix from the CCP-EM suite\cite{26-28}.

RNA release assays were performed where a constant amount of virus was mixed with fJAM-A of varying concentrations at various pH values alongside a nucleic acid binding dye (Syto9; ThermoFisher Scientific) and fluorescence measured every five minutes for four hours using a plate reader (BMG Labtech) \cite{11}.

Negative staining of samples was performed by washing the grids in deionised water following incubation of the sample on the grid for two minutes. The grids were then washed and incubated in 2% uranyl acetate for two minutes prior to visualisation in a JEOL 1200 EXII transmission electron microscope equipped with a Gatan Orius charge coupled device camera.

RESULTS / DISCUSSION
Feline calicivirus particles were purified and combined with an equal amount of fJAM-A soluble ectodomain at 4°C. Either FCV only or FCV decorated with the soluble ectodomain of fJAM-A were then imaged in a Titan Krios and the resulting micrographs processed as described above.

The three-dimensional structure of FCV (see Figure 1A) shows the characteristic cup shaped depressions that can be seen on the surface of all caliciviruses, that are formed by the VP1 P domains\cite{2, 11, 18-20, 29, 30}. Upon receptor engagement, additional density is visible above each of the P domains of VP1 which corresponds to fJAM-A (see Figure 1B). We have shown that the soluble ectodomain of fJAM-A forms a dimeric complex in solution and, unsurprisingly, our cryoEM data show that two fJAM-A proteins appear to bind to two VP1 capsid proteins although, surprisingly, the ‘usual’ dimeric interface between these two fJAM-A proteins is disrupted/altered upon virus binding. Despite the FCV binding site occurring away from the fJAM-A cis-dimerisation site, fJAM-A proteins appear to bind to VP1 proteins in a head-to-tail arrangement rather than their native arrangement (both in solution and their predicted conformation at tight junctions between cells)\cite{11}. Upon fJAM-A binding to the FCV capsid, the VP1 capsomeres undergo conformational changes including a 15° anti-clockwise rotation of the A/B dimers and the tilting of the C/C dimers away from the two-fold symmetry axes. These conformational changes result in the breakage of icosahedral symmetry and thus a smearing of the density in these regions (see Figure 1D and F).

Due to this loss of icosahedral symmetry in the virus, a relatively novel method of image analysis was utilised known as focussed classification\cite{11, 23-25}. Briefly, focussed classification allows the 3D classification and reconstruction of specified regions of the assembly (rather than the whole complex). Due to the loss of symmetry at the 2-fold symmetry axes in fJAM-A decorated FCV, we focussed the classification on the C/C VP1 dimers (2-fold symmetry axes). Upon inspection of the different classes, one class contained additional density visible at the edge of the area corresponding to the 3-fold symmetry axis which was previously unseen. Subsequent classification was focussed on the 3-fold symmetry axes of the decorated virus and revealed a novel virus feature in a subset corresponding

FIGURE 1 Feline calicivirus undergoes a conformational change upon receptor engagement. Structures of feline calicivirus undecorated (a, c, e) and decorated with the soluble ectodomain of fJAM-A (b, d, f) are shown. Three dimensional maps of the two virus structures are presented (a and b) coloured by radius alongside central sections of the reconstructions (c and d) showing the additional blurred density on each of the capsid spikes (d). Spherical sections for the two structures are also shown (e and f) to highlight to rotation of the spikes (pink boxes) upon fJAM-A binding (f).
A: VP1 capsid proteins are shown under both low pH (pH3) and neutral pH conditions. Scale bars of 200nm are depicted.

FIGURE 3. Negative staining of feline calicivirus with comparable amounts of fJAM-A reveals viral disassembly under low pH conditions. Samples of feline calicivirus alone or incubated with varying ratios of fJAM-A reveal viral disassembly under low pH conditions. As each virus particle is formed of 180 VP1 proteins, a rough estimate of receptor/fJAM-A engagement required for the observed conformational changes can be calculated as 16 receptor molecules per virus particle. As FCV enters host cells by clathrin mediated endocytosis, it is likely that the virus binds to additional fJAM-A proteins during endocytic uptake, allowing endosomal escape [11, 31]. To further investigate this, a constant amount of FCV was incubated with varying amounts of fJAM-A in the presence of a nucleic acid dye which fluoresces upon binding to RNA (or DNA). As endosomal acidification is necessary for FCV entry into host cells, this assay was performed at a range of pH values from 3 to 9 and showed the release of viral RNA only in the low pH conditions (pH 3 or 4) and only when a certain amount of receptor/fJAM-A was present (data not shown) [25, 36]. To further investigate the amount of fJAM-A required to cause the conformational changes in FCV (and therefore release of the viral RNA at low pH), we performed negative staining electron microscopy of FCV with varying ratios of fJAM-A at neutral and low pH. As shown in Figure 3, no intact FCV particles were visible upon incubation with an equal amount of fJAM-A at low pH, however, intact particles are present at neutral pH (under conditions utilised for cryo-EM described above). Upon incubation of FCV with a 1:11 ratio of fJAM-A:VP1, a limited number of broken and irregular virus particles were visible. However, when the virus was incubated with a 1:12 ratio, mostly intact virus particles were seen (see Figure 3), suggesting that a ratio of 1:11 fJAM-A:VP1 capsid proteins is the minimum required to cause viral disassembly at low pH (and by inference, cause the conformational changes in the capsid; see Figure 1) [11]. As each virus particle is formed of 180 VP1 proteins, a rough estimate of receptor/fJAM-A engagement required for the observed conformational changes can be calculated as 16 receptor molecules per virus particle.

SUMMARY AND CONCLUSIONS
Upon engagement with its cellular receptor, fJAM-A, feline calicivirus undergoes a conformational change resulting in the rotation and tilting of the virus spikes. This tilting causes breakage of the viral icosahedral symmetry so focussed classification was employed to further characterise these changes. This asymmetric analysis resulted in the discovery of a novel portal or tunnel-like structure formed by a virus which likely engages into the endosomal membrane to mediate genome delivery and allow infection to proceed.
Fast volume imaging with bessel beam tomography and machine learning

INTRODUCTION
Fluorescence microscopy is one of the most widely used tools for investigating the dynamics of biological systems. Such dynamics, for example the propagation of neural signals in the brain, often plays out in all three spatial dimensions. This is challenging for microscopy which typically only monitors a single two-dimensional focal plane at a time. To address this problem, we developed a fluorescence tomography approach that allows recording volume information in a single frame scan from sparsely labeled samples.

Optical microscopes typically record from a single focal plane at a time. This can be limiting for samples that show fast dynamics across multiple focal planes, which is often the case for example when monitoring neural activity in the brain at cellular resolution. In this situation, neurons are arranged in three dimensions and the dynamics of these cell ensembles evolves over a volume. To monitor such dynamics it can be advantageous to sacrifice some spatial resolution for temporal resolution. We describe in the following one such approach that can reconstruct volumetric information from images recorded in a single frame scan with multiple laser beams.

TOMOGRAPHIC IMAGING
The developed approach relies on tomography, which reconstructs volume information from multiple two-dimensional projections. Tomographic imaging in optical microscopes can be achieved using scattered light or using fluorescence. In the latter case the sample is typically rotated with respect to the imaging path to obtain multiple projections. However, this method is not compatible with many imaging situations where the sample can only be accessed from one side with a single microscope objective and is not transparent, as is typically the case for in vivo imaging in neuroscience.

TEMPORALLY MULTIPLEXED BESSEL BEAMS
For generating projections under these conditions, one can take advantage of Bessel beams. Instead of a focal spot, these beams generate a focal line that is extended along the optical axis while maintaining a lateral resolution that is similar to a focused Gaussian beam; Bessel beams have for example been applied for in vivo imaging of neural activity of sparsely labeled samples. By introducing a lateral offset when passing through the microscope objective these Bessel beams can be tilted by an angle $\theta$ with

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FIGURE 1
a) Side projections allow undistorted 3D reconstruction. b) Projections under an oblique angle lead to distorted 3D reconstructions. c) Steps in the volumetric reconstruction process.
Machine learning aided bessel beam tomography

BEVBEL BEAM TOMOGRAPHY

As described in more detail below, images were reconstructed in the following steps: First, four 2D projections from a volumetric sample were recorded by scanning four temporally multiplexed Bessel beams, each with a different projection angle; then, similar to computed tomography, images were reconstructed using so-called back projection, which computationally inverts the projection signal generation process. Compared to reconstruction using side projections (fig. 1a), which would be best suited for tomography, the oblique projection angles lead to distortions of the reconstructed volume (fig. 1b). Then, again taking advantage of image reconstruction approaches developed for computed tomography, these distortions were finally corrected using machine learning, by applying a suitable convolutional neural network. These steps are summarized in figure 1c.

TOMOGRAPHIC RECONSTRUCTION

Bessel beam tomography first requires generating four beams with different orientations. Here, the elevation angle was kept constant for all beams (about 13 degrees, limited by the numerical aperture of the objective), and the azimuth was varied in steps of 90 degrees. In a calibration step the exact orientation of the Bessel beams across respect to the optical axis; scanning across the sample then results in a projection along this tilted beam axis. For volumetric reconstruction, multiple such projections need to be recorded from different angles and to additionally cope with fast sample dynamics, these projections have to be recorded at the same time. This can be achieved using temporal multiplexing where, using pulsed fluorescence excitation together with time-gated detection, signals generated by different beams can be sorted into different channels [6].

The volume reconstructions obtained from these oblique projections leads to a distortion along the z-axis with a factor of 1/sin(θ), as shown in figure 1b. To correct these distortions we used machine learning where a convolutional neural network (U-net) was trained using simulated data. Using a large set of simulated ground truth data as well as the corresponding simulated projection images such a network can be trained to recover the volume at high resolution based on the projection images. Figure 3 shows the reconstruction of four projections experimentally recorded from a sample of pollen grains (fig. 3a). Applying back projection to the four images leads to a distorted volume, as shown in the first row of figure 3b. Applying the trained U-net to these back projections can partially restore the information in the z-axis (second row in figure 3b) as seen in the comparison with the ground truth recorded using two-photon microscopy with a Gaussian beam in the third row of figure 3b.

CONCLUSION

We combined scanning microscopy with tilted Bessel beams, temporal multiplexing and tomographic reconstruction aided by machine learning to reconstruct volumetric information recorded in a single scan across a three-dimensional sample. This allowed recording volume information in a single frame scan. In future work optimizing the tilted beams, for example using objectives with higher numerical aperture, as well as training neural networks with larger training data sets specific for the sample of interest will allow improving the spatial and temporal resolution of this imaging approach.

FIGURE 1

Reconstruction of beads. a) Four recorded projections with each Bessel beam from a volumetric sample composed of 1 μm diameter beads. b) Reconstruction of the sample using back projections compared to ground truth.

FIGURE 2

Reconstruction of pollen grains. a) Four projections recorded with Bessel beams from a sample of pollen grains. b) First row: reconstruction of the sample using only back projection. Second row: reconstruction of the sample after applying a U-net to the back projections to correct distortions. Third row: ground truth recorded with a two-photon microscope with a Gaussian focus.

FIGURE 3

Reconstruction of pollen grains. a) Four projections recorded with Bessel beams from a sample of pollen grains. b) First row: reconstruction of the sample using only back projection. Second row: reconstruction of the sample after applying a U-net to the back projections to correct distortions. Third row: ground truth recorded with a two-photon microscope with a Gaussian focus.

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A view of the dark side

Late last year, a team of chemists from Cornell University, US, unveiled a super-resolution method to image non-fluorescent reactions at nanometre resolution.

Based on widefield fluorescence microscopy, so-called COMPEITS - COMPetition Enabled Imaging Technique with Super-resolution - relies on competition between fluorescent and non-fluorescent reactions, as part of a single-molecule fluorescent-detection scheme. Crucially, the new method can be used to image a host of non-fluorescent reactions, critical to water decontamination, industrial chemical separation processes and more.

"This method turned out to be actually quite simple to implement," highlights Professor Peng Chen from the Department of Chemistry and Chemical Biology, Cornell. "[It] can be broadly applied to image various classes of non-fluorescent systems, such as unlabelled proteins, neurotransmitters and chemical warfare agents... and really extends reaction imaging to an almost unlimited number of reactions."

"We expect it to have profound impacts on many fields including energy science, cell biology, neuroscience and nanotechnology," he adds.

Without a doubt, a super-resolution fluorescence microscopy methods, such as stimulation emission depletion microscopy and photoactivated localisation microscopy, have revolutionised biology and other research fields. However, these techniques can only interrogate particles and processes that fluoresce or are fluorescently labelled, which doesn't help the lion-share of biological and chemical reactions.

Given this, Chen's group set out to develop a super resolution method that can interrogate non-fluorescent processes. As Chen's colleague, Dr Xianwen Mao, tells Microscopy and Analysis: "The inability of original super-resolution techniques to study non-fluorescent reactions has been a primary limitation for quite some time as the reactants and products in so many reactions are completely non-fluorescent."

"So we wanted to tackle this long-standing challenge and develop a super-resolution technique with much broader applicability," he adds.

Chen's research group has spent many years pioneering the application of single-molecule fluorescence imaging to observe chemical reactions in real time. As part of this, the researchers have been looking at single molecule catalysis alongside single-nanoparticle catalysis, reactivity and dynamics. And what's more, the researchers have developed new super-resolution methods to, say, track single molecule processes, such as DNA kinetics in living cells, and map photoelectrocatalytic reactions, including water-splitting, to sub-particle resolution.

So this time around, the researchers decided to harness their expertise on single-molecule reactions, and developed COMPEITS. The new method uses an inverted Olympus IX71 fluorescence microscope with a photoelectrocatalytic microfluidic cell as well as two-laser epifluorescence illumination to drive and image a reaction. Within the flow cell, a catalyst particle is used to catalyse two reactions - the all-important non-fluorescent reaction and its auxiliary fluorescent partner reaction. As the fluorogenic reaction proceeds, its product molecules fluoresce and can be imaged at nanometre resolution. However, as the non-fluorescent reaction also gets underway, it competes for the same surface sites on the catalyst particle, suppressing the rate of the fluorogenic reaction. According to the researchers, the extent of this suppression can be imaged using the widefield fluorescence microscope set-up to nanometre resolution, delivering super-resolution spatial information on the non-fluorescent reaction.

"We introduce the competing agents into the photoelectrochemical microfluidic cell [with the photocatalyst] so we can then visualise the reactions," says Mao. "We can, for example, apply potential to modulate the electric field and change the photoelectrocatalytic efficiencies, and we can also use one of the lasers to generate the charge carriers in the photocatalyst and drive the reactions."

"This took around two years to develop, and this imaging capability is realised by combining the epifluorescence microscopy with the microfluidic cell, and excitation and imaging lasers," he adds. "By doing this, we have all of these capabilities."

The researchers have already used COMPEITS to image the photoelectrocatalytic oxidation of hydroquinone, a micro pollutant found in aquatic ecosystems, on single bismuth vanadate photocatalyst particles in exquisite detail. They were able to map, with nanometre precision, the non-fluorescent surface reaction, discovering previously unknown behaviours of the catalyst. Indeed, they could see that hydroquinone adsorption varied.

‘COMPEITS’ can image non-fluorescent reactions at nanometre resolution – meet the researchers pioneering single-molecule fluorescence microscopy, reports Rebecca Pool.

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**DURING A FLUOROGONIC oxidation reaction, fluorescence microscopy can identify a catalyst’s most active sites (left, red and yellow). Adding a competing non-fluorescent reaction decreases the reaction rate (centre) and reveals where on the catalyst a “dark” reaction occurs (right).**

**No Competition**

**With Competition**

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**COMPEITS Image**

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**DR XIANWEN MAO and Professor Peng Chen, Cornell, have developed COMPEITS to image non-fluorescent reactions.**

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Lab focus
Interrogating systems

Professor Peng Chen and colleagues have used single-molecule imaging to interrogate a host of nanoscale materials and biological systems, employing single-molecule fluorescence imaging, single-molecule FRET, single-molecule tracking, super-resolution localisation microscopy and magnetic tweezers.

More than a decade ago, Chen and colleagues were using single-molecule fluorescence microscopy, on their homebuilt Olympus IX71 total internal reflection microscope, to study the catalysis of individual gold nanoparticles. They also managed to pinpoint the reactive sites on single-walled carbon nanotube electrocatalysts.

More recently, Chen and colleagues have used magnetic tweezers, mounted onto an Olympus IX71 inverted microscope, to study polymer growth, in real-time and at the single-polymer level. After fixing the polymer chain to a glass plate and then applying a ruthenium catalyst to trigger growth, the researchers were able to use the tweezers to pull the chain and watch it grow.

Around the same time, the researchers used single-molecule super-resolution catalysis imaging in correlation with electron microscopy to visualise enhanced catalytic activity at the palladium-gold interface in single bimetallic nanoparticles. As Chen said at the time: “This is experimentally observing something that people knew about but couldn’t see, and now we have a new way of directly seeing it.”

And only last year, Chen and colleagues won $2 million from the US Department of Energy to study how light absorbing quantum dots can be combined with bacteria for efficient energy conversion. Heading up an interdisciplinary team of researchers, including Professors Tobias Hanrath and Professor Buz Barstow also from Cornell, Chen will create a hybrid quantum dot-bacterial cell system that can harvest sunlight and use carbon dioxide to produce high-value chemicals including plastic precursors, the biofuel butanol and the bio-plastic, polyhydroxybutyrate.

As Chen points out: “This is a system that others have shown to work, and it’s very promising, but it hasn’t been brought to large scale or with extremely high efficiencies yet.”

“We need to understand how the system works at a fundamental level, and find the underlying factors that can contribute to or limit system performance, so we can potentially engineer or improve it,” he adds.
4D STEM with a direct electron detector

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INTRODUCTION

Scanning transmission electron microscopy (STEM) is a powerful tool for studying specimens at very high (sub-angstrom) spatial resolution. Conventional detectors for STEM, such as bright-field (BF), annular bright-field (ABF), annular dark-field (ADF) and high angle annular dark-field (HAADF) detectors are monolithic, and form images, (I_k, y), by integrating the total signal over a range of scattering angles in the diffraction plane as the focused STEM probe is rastered over a two-dimensional (2D) area of a specimen (Figure 1a). In contrast, detectors for four-dimensional STEM (4D STEM) record a pixelated, angle-resolved 2D convergent beam electron diffraction (CBED) pattern, (I_k, k_x, k_y, y), for every position, (x, y), in the 2D STEM raster over the specimen (Figure 1b), yielding a 4D dataset. As with any diffraction pattern in STEM, the angles in the diffraction plane sampled in 4D STEM may be controlled by varying the microscope camera length and convergence angle will depend on the types of data that one wishes to extract from the 4D STEM dataset.

The key advantage of 4D STEM compared to conventional 2D STEM imaging is that, in a single scan, one can acquire a 4D dataset that contains all possible 2D STEM images of the sample, as well as a variety of novel signals that cannot be derived from conventional 2D STEM imaging. The speed and sensitivity of 4D data collection can be improved by employing a direct electron detector, which can either be used as a stand-alone detector or can be used alongside conventional STEM detectors. For example, a 4D STEM detector can work in conjunction with a traditional annular detector, which can record a 2D image using the signal from a region of the diffraction plane outside of that recorded by the 4D STEM detector. This flexibility allows users to upgrade their existing STEM instruments with 4D STEM capability.

Here, we give a basic overview of the principles of 4D STEM, including some of the types of images that can be extracted from 4D STEM datasets, as well as an overview of the types of direct detectors that can be used for 4D STEM. A complete review of the technique, including historical developments may be found in Ref. [1].

4D STEM DETECTORS

The availability of high-performance electron detectors has been one of the main catalysts for the growing popularity of 4D STEM. Early efforts at recording 4D datasets consisting of CBED patterns were limited by the relatively slow readout speeds of CCDs[2,3]. Recording 4D STEM data is considerably more practical when using a sensitive detector capable of millisecond or sub-millisecond readout speeds. Although 4D STEM is possible using a fast scintillator coupled camera, direct electron detectors are particularly well-suited to 4D STEM due to their high detective quantum efficiency (DQE) and high signal to noise ratio (SNR) compared to scintillator coupled cameras.

There are two principal types of direct electron detector that have been used for 4D STEM applications, and both types have different strengths and weaknesses. A hybrid pixel array detector (PAD) is a hybrid integrating and counting technique’s dynamic range from 4D datasets and how the technique’s dynamic range requirements can be met using a sensitive detector capable of millisecond or sub-millisecond readout speeds. Although 4D STEM is possible using a fast scintillator coupled camera, direct electron detectors are particularly well-suited to 4D STEM due to their high detective quantum efficiency (DQE) and high signal to noise ratio (SNR) compared to scintillator coupled cameras.

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BIOGRAPHY

Barnaby Levin is a Materials Applications Scientist at Direct Electron. He studied Physics as an undergraduate at the University of Oxford before completing a Ph.D. in Applied Physics at Cornell University in 2017, under the supervision of David Muller. Barnaby completed a Postdoc at Arizona State University, supervised by Peter Crozier, before joining Direct Electron in November 2019. Barnaby’s responsibilities at Direct Electron include supporting customers with 4D STEM applications.

ABSTRACT

The recent development of fast, sensitive, pixelated detectors has led to the growing popularity of four-dimensional scanning transmission electron microscopy (4D STEM). 4D STEM involves acquiring a 2D convergent beam electron diffraction pattern, at every pixel of a 2D STEM raster, generating a 4D dataset containing a vast amount of information about the specimen. Here, we describe the basic principles of 4D STEM, including different types of information that can be extracted from 4D datasets and how the technique’s dynamic range requirements can be met using a hybrid integrating and counting Monolithic Active Pixel Sensor (MAPS) Direct Detection Device (DDD®).

FIGURE 1

Schematic diagrams illustrating conventional STEM and 4D STEM. a) In conventional STEM, monolithic detectors are used to produce images by integrating a signal from different angular regions of the diffraction plane. b) In 4D STEM, an entire 2D CBED pattern is recorded at each probe position of a 2D STEM raster, resulting in a 4D dataset.

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pixel array detector (EMPAD) described in Ref. [7] (Thermo Fisher Scientific, Waltham, MA USA) bump-bonded to an application specific integrated circuit. PADs typically exhibit extremely high dynamic range, due to their high saturation value per pixel[3], but their relatively small number of pixels limits the angular resolution of the detector and limits the ability to use the PAD as an “all-in-one” detector that can be used for other TEM imaging applications, where a large number of pixels is advantageous. In contrast, monolithic active pixel sensors (MAPS) use relatively thin pixels, which limits beam spreading within each pixel, allowing for smaller pixel sizes (e.g. 6.5 x 6.5 µm in the case of a DE-16 (Direct Electron, San Diego, CA USA)) and a correspondingly much larger number of pixels per sensor. The traditional weakness of MAPS detectors for 4D STEM is their relatively limited dynamic range, due to a relatively low saturation value per pixel. This can be mitigated to some degree by using the large number of pixels on the sensor to spread features of interest in the CBED pattern over many pixels.

In many 4D-STEM experiments, it is desirable to simultaneously record very bright and faint features in the CBED pattern without saturating the detector. For example, one may wish to simultaneously record the bright central (0,0) beam (bright-field disc), as well as the faint signal of scattered electrons in the dark-field region. To maximize the signal-to-noise ratio of the sparse dark-field region, MAPS detectors can be used to perform electron counting[16,17], which excludes background noise and normalizes the signal of each detected electron. However, isolating and counting each primary electron incident on the detector requires sparsity (i.e. < ~0.1 e- per pixel), which is generally not satisfied in the intense bright-field disc. Hybrid counting and integrating techniques can provide a method of overcoming the challenge of limited dynamic range for 4D STEM using MAPS detectors[18,19]. Direct Electron has developed a patent-pending hybrid counting technique[20], which is implemented for our DE-16 detector. The workflow of the method is illustrated in Figure 2. During 4D STEM data acquisition, specialized software (DE-4DExplorer) is used to calculate a sparsity map for each frame. The sparsity map is a binary mask corresponding to regions of the frame where the number of primary electrons per pixel is low enough to be processed using electron counting. In these sparse regions (primarily in the dark-field area of the diffraction pattern), individual primary electron events can be distinguished and thus electron counting can be used to improve SNR by effectively eliminating the Landau noise caused by the variable amount of energy deposited on the sensor by each primary electron[16,17]. In bright regions (such as the bright-field disc), there is a sufficient number of primary electrons to statistically diminish Landau noise. However, in these bright areas many primary electrons are recorded by the same or neighbouring pixels, making it impossible to distinguish individual electron events using electron counting algorithms. To maximize the SNR without sacrificing linearity, the hybrid counting technique performs electron counting in the sparse regions of the CBED pattern while using conventional integration in the non-sparse regions. The intensity of non-sparse regions is scaled based on the average pixel intensity per primary electron. Thus, the intensity value for each pixel in the final processed frame approximately corresponds to the actual number of primary electrons incident on each pixel in each frame. By applying this hybrid counting technique, the DE-16 can image the low-angle dark-field regions of the CBED pattern with a high SNR, while simultaneously recording useful information from the more intense bright-field disc.

Note that Direct Electron’s hybrid counting method automatically detects sparse regions in each recorded camera frame without prior information from the user. Electron counting is performed without manually having to define the position and size of the bright and sparse regions in advance. In principle, hybrid counting may therefore be applicable not only to 4D STEM, but also to other techniques that require high sensitivity and high dynamic range, such as electron energy loss spectroscopy (EELS).

4D STEM DATA ANALYSIS

4D STEM datasets occupy far more computer memory than a conventional STEM image. For example, a 4D STEM dataset consisting of a 1024x1024 pixel CBED pattern recorded at every pixel of a 1024x1024 STEM raster and stored in 16-bit format will occupy ~2 GB of memory. The acquisition of 4D STEM data also requires very high rates of data transfer compared to 2D STEM imaging. For example, a data transfer rate of ~2 GB/s is required for 4D STEM acquisition of a 1024x1024 pixel CBED patterns at a rate of one frame per millisecond. In practice, the memory occupied by a 4D STEM dataset may be reduced either by limiting the number of spatial pixels in the STEM raster, by using fewer pixels to record patterns in diffraction space, or by downsampling the data after acquisition.

Analysis of 4D STEM datasets typically requires a powerful computer and specialized software. The data presented below has been analyzed using a combination of custom written Python code, as well as libraries from Hyspy[21] and py4DSTEM[22]. Other software tools currently available for the analysis of 4D STEM data include LibTEM[23], Pycroscopy[24], pixiSTEM[25], pyKemi[26] and the Cornell Spectrum Image plugin for ImageJ[27], which was originally written for the analysis of hyperspectral data, but now also includes 4D tools.

One of the simplest operations that can be applied to the 4D dataset is to mask an area of diffraction space and integrate the signal from within the mask in each diffraction pattern to recover a 2D STEM image[28,29]. This is illustrated in Figure 3 for a 4D STEM dataset recorded from a sample of SrTiO$_3$ imaged along a [001] direction. Using this masking technique, images equivalent to that of any conventional STEM detector (e.g. BF, ABF, AD) can be generated, but with the advantage that the 4D STEM dataset allows complete flexibility in choosing detector angles. Indeed, one can vary the shape and position of the mask to generate images from any kind of “virtual detector”[25,27]. For example, one can choose to reduce the convergence angle of the STEM probe such that Bragg discs are well separated and do not overlap. In this mode, diffraction patterns are often referred to as nanobeam electron diffraction (NBED) or nanobeam diffraction (NBD) patterns, rather than CBED patterns. One can then generate an image analogous to that of dark-field TEM by placing a circular mask around a diffracted Bragg disc in the NBED pattern, with the mask acting as a virtual objective aperture[28,29].

In addition to reconstructing images using simple masks, 4D STEM enables the reconstruction of a differential phase contrast (DPC) images[23,24,30], which were previously only available by using segmented detectors[31,32], and “Centre of Mass” (COM) images[33,34], which are unique to 4D STEM, and have also been referred to as “first moment” or COM DPC images in the literature[35,36]. DPC and COM both
and as to electric fields in the specimen[23,29-34], which can cause a shift in the average momentum of the electron probe either by displacing the entire diffraction pattern, or altering the distribution of intensity within the diffraction pattern, depending on the relative length scales of the probe and the source of the field[25]. Magnetic fields can also be detected using DPC and COM when operating the microscope in a Lorentz field[24,33,34,52]. An example of COM imaging is shown in Figure 4. For a diffraction pattern consisting of pixels with coordinates $k = (k_x, k_y)$, and with the intensity in each pixel defined as $I$, the position of the centre of mass in the diffraction pattern can be defined mathematically as:

$$k_{COM} = \frac{\int k I(k) \, dk}{\int I(k) \, dk}$$

A suitable choice of origin for measuring $k_{COM}$ is the position of the centre of the probe in vacuum when no specimen is present, which under ideal conditions should correspond to the centre of the optic axis and the centre of the detector. The effect of the nuclear potential on the COM is apparent when imaging a atom at atomic resolution[22,23,47-49]. Figures 4a and 4b show the magnitude and direction respectively of the shift of the COM for the same area of the SrTiO$_3$ sample displayed in the images in Figure 3. The magnitude of the shift of the COM is strongest when the probe is close to, but not directly on, the Sr and Ti/O columns, and the direction of the shift is oriented towards the columns. Here, the COM images are measuring the deflection of the negatively charged electron beam by the screened, positively charged atomic nuclei.

As with the generation of conventional STEM images from 4D STEM data, one has complete flexibility to apply a mask of any shape or size to the 4D dataset when generating DPC or COM images, enabling the DPC or COM associated with specific regions of the CBED or NBED pattern to be calculated[27].

Another application of 4D STEM is mapping local changes in lattice constant and local strain within the specimen[28,33]. Strain information can be extracted from 4D STEM datasets in different ways. When acquiring 4D STEM data containing NBED patterns with well separated Bragg discs, strain is encoded in the disc positions[28], or for very thin samples the disc centre of mass[33]. For polycrystalline or amorphous materials, the ellipticity of NBED patterns may also be useful[29].

Further techniques that make use of 4D STEM datasets include mapping of crystal grain orientations in a specimen[25,43-47], where there is some overlap between 4D STEM and the similar technique of nanobeam electron diffraction[28], and electron ptychography, in which computational methods are used to reconstruct the projected potential of the specimen, with the potential for spatial resolution beyond that of traditional STEM imaging techniques[29-31]. A number of other imaging modes besides those mentioned above may also be performed using 4D STEM[53]. As 4D STEM is a relatively new and growing technique, there may be further imaging modes that are yet to be developed that can take advantage of the wealth of information contained in 4D STEM datasets.

MATERIALS AND METHODS

The data presented in this article was acquired on an Aberration Corrected FEI/Thermo Scientific Titan 80-200 STEM (Thermo Fisher Scientific, Waltham, MA USA), operated at 200 kV with a STEM probe current of ~20 pA and a probe convergence semi-angle of 24.5 mrad. A DE-16 direct detection camera (Direct Electron, San Diego, CA USA) was used to record 4D STEM data. Each of the CBED patterns in the 4D STEM data was captured using a 1024x1024 pixel centred area of the 4096x4096 pixel sensor. A hardware binning of 2 was applied to the CBED patterns, reducing them to 512x512 pixels in size, allowing the 4D STEM dataset to be recorded at a rate of ~1100 frames per second (fps). Higher frame rates (up to >4200 fps) are accessible by further reducing readout area.

**SUMMARY AND CONCLUSIONS**

In summary, 4D STEM is a versatile technique that enables a multitude of different types of analyses to be performed on a specimen. These include reconstructing images equivalent to those of any traditional STEM detector of any shape or size, imaging of electric and magnetic fields, mapping strain within a specimen, mapping crystal grain orientations, analyzing medium range order, and generating ptychographic reconstructions with high spatial resolution. Both hybrid PDAs and MAPS detectors may be used for 4D STEM. Using a hybrid counting method, implemented on detectors such as the DE-16, dynamic range and sensitivity are sufficient to allow simultaneous imaging of the bright-field disc and low-angle dark-field regions, overcoming a traditional weakness of the MAPS architecture, allowing users to record 4D STEM datasets that take advantage of the angular resolution available to a detector with a high pixel count. 4D STEM methods using pixelated direct electron detectors can complement existing STEM detectors and can readily be implemented on existing STEM instruments.

FIGURE 3  a) A position-averaged CBED (PACBED) pattern, derived from a 4D STEM dataset acquired from a SrTiO$_3$ sample, imaged along a [001] direction. b) Diagram of the crystal structure of SrTiO$_3$ in a [001] projection. Example of a mask that can be applied to the 4D dataset to produce a BF image. c) Example of a mask that can be applied to the 4D dataset to produce an ABF image. d) Example of a mask that can be applied to the 4D dataset to produce an ABF image. The crystal diagram in the lower corner of each image indicates atomic column positions. The spacing between adjacent Sr atomic columns (green) is ~3.9 Å.

FIGURE 4  a) Image showing the magnitude of COM shifts in a 4D STEM dataset acquired from a sample of SrTiO$_3$. The target COM shifts occur when the probe is adjacent to an atomic column. The diagram beneath the image illustrates how the magnitude and direction of the shift in the COM are defined with respect to the position of the centre of the probe in vacuum. b) Map showing the direction of COM shifts from the same dataset as in (a). When the probe is positioned adjacent to an atomic column, the COM shifts towards the column. The direction indicated by each colour is shown by the colour wheel beneath the image.---
Mid-November in the UK Cryomicroscopy community is synonymous with the annual Cryo Microscopy Group (CMG) meeting. November 2019 was no different and saw a diverse line up of speakers share their latest achievements at the University of Nottingham with a receptive and enthusiastic audience.

The first speaker Thomas Braun from the University of Basel presented his work on revolutionary cryo-EM grid preparation which uses nanolitre deposition onto EM grids for the purpose of preparing thin films of solutions. The use of a microcapillary technology for the deposition and microfluidics for solution mixing and sample extraction combine to give a very powerful sample preparation technique. Braun presented this using the title single-cell proteomics in which he showed it was possible to extract the proteins by cryo-TEM and single particle analysis.

Following on from Braun, Nicole Hondrow from the LEMAS centre at the University of Leeds, provided the audience with a wonderful overview of her work using cryo techniques for materials systems characterisations. She explained that she’d started using Cryo-TEM to analyse nanoparticle materials systems characterisations. Of her work using cryo techniques for the audience with a wonderful overview the University of Leeds, provided the latest achievements at the University of Nottingham with a receptive and enthusiastic audience.

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**OptoSplit II bypass – 2-way emission image splitter**  
Applications: Simultaneous multi fluorescent probe imaging, FRET, simultaneous dual depth imaging, TIRF, Super resolution  
Key features: • 1 or 2 images on a single camera chip • Supports 25mm filters • Supports for sensors up to 18.8mm diagonal • Single lever switching from split to bypass • 7225mm to 875nm coatings on all surfaces

**TwinCam – multi camera adapter**  
Applications: Simultaneous use of two dyes or genetic markers, FRET, ratiometric calcium, simultaneous multi depth imaging, super resolution  
Key features: • 2 cameras on 1 microscope port • Supports for sensors up to 18.8mm diagonal • 2425mm to 875nm AR coatings on all surfaces • 25mm filters and standard microscope dichroic sizes • Compact design with integral C-mount input

**Triplesplit – 3-way emission image splitter**  
Applications: FRET, ratiometric calcium, simultaneous multi depth imaging, super resolution  
Key features: • 1 or 3 images on a single camera • Support for sensors up to 25mm diagonal • 2425mm to 875nm coatings on all surfaces • Simple and precise controls for image registration • Supports 25mm filters and ultra flat dichroics

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**DE-16 Camera System**  
In-situ TEM, 4D, STEM materials science, biological, Cyo-EM  
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Leica and the European Molecular Biology Laboratory have signed an open innovation collaboration agreement in which Leica will support the upcoming EMBL Imaging Centre, currently under construction at the EMBL campus in Heidelberg, Germany.

The signed framework agreement, which was negotiated and concluded by EMBLEM - EMBL’s wholly-owned commercial technology transfer subsidiary - allows for a broad and long-term partnership.

According to Leica, users will be able to test new technologies for applications in their research much earlier and organisations can feed these experiences back into their technology development, to make sure new products deliver what users need to accomplish their research goals.

As Markus Lusser, president of Leica Microsystems, says: “The direct exchange of developers and researchers will pave the way for breakthrough applications, ones that confirm their relevance for state-of-the-art scientific research right from the start.”

The framework agreement allows an even stronger interlinking between the early stages of new technology development and forefront scientific work.

As part of the agreement, the EMBL Imaging Centre will house cutting edge microscope equipment from Leica, which will be supported by Leica engineers to support the users of the EMBL Imaging Centre in their research.

The EMBL Imaging Centre is scheduled to open in mid-2021. It will host up to 300 visiting scientists per year, giving them access to the very latest imaging technologies.

Linkam has launched a latest update to its cryo-stage for correlative microscopy; CMS196V 3. The CMS196V 3 is a cryo-correlative microscopy system enabling the full workflow of Correlative Light and Electron Microscopy (CLEM).

It is said to be ideal for the correlation of high resolution structural information with biochemical processes within cells.

The latest model can be integrated with a wide range of research grade upright microscopes, offers enhanced sample stability for cryo-imaging, improved sample handling and reduced sample contamination.

The CMS196V 3 maintains the vitrified state of the sample by means of liquid nitrogen cooling and provides proven capabilities to safely handle and transfer cryo-samples and image them with optical microscopy.

Samples are kept free of contamination at all times due to active self-cleaning via the liquid nitrogen cold trap.

The integrated, encoded, motorised XY stage enables coordinate mapping required to locate the same sample position in the fluorescence microscope, as well as in the EM. The chamber top-up keeps samples vitrified at a constant -196°C, reducing photo bleaching and maintaining structural integrity of samples.

The sample cassette holder can hold up to 3 grids and ensures contamination-free sample loading, storage and transfer. Cassettes are available for different grid types including FEI, Planchette, Bessey, Polara and custom designs.

Optional Liquid Nitrogen autofill can extend the use of the system for up to six hours, unattended.

The LINK software for the CMS196V 3 provides control and monitoring of the system. When combined with the optional high sensitivity camera and imaging module, LINK enables fully automated, tiled, image capture.

The system produces a single, tiled, image of the full EM grid at high resolution. This can then be used to navigate the sample and save co-ordinates of areas of interest.

A full SDK is available for users to develop and integrate control of the CMS196V 3 into their own applications. Support for the CMS196 V 3 is also provided with Zeiss ZEN, Nikon NIS-Elements software as well LabVIEW.
Coxem has added the EM-30N to its EM-30 family of tabletop microscopes. The new microscope features re-designed imaging electronics for improved resolution with less noise at high magnification, and also introduces a new Panorama mode for wide area scanning.

Delivered with both SE and BSE detectors, the EM-30N allows the operator to view either detector individually, side-by-side, or as a composite image for a better understanding of microstructure and chemistry.

Low Vacuum mode allows the operator to image non-conductive samples without any special pre-treatment, while a built-in optical camera helps to manage multi-sample holders and simplify navigation for users.

Powered by NanoStation software, the EM-30N is said to seamlessly integrate all SEM functions with EDS routines such as compositional particle analysis.

It provides advanced image analysis tools, such as line profile analysis, to more accurately determine particle size when working at the nanoscale. Automated features, such as near-real-time automatic brightness/contrast, simplify operation and help novice users achieve consistent results. An optional STEM detector allows the EM-30N to utilize its 30 kV accelerating voltage capability to perform TEM analysis of samples on standard TEM grids, while the optional CoolStage allows analysis of samples from -25°C to 50°C.

Thermo Fisher Scientific has unveiled the Aquilos 2 Cryo-FIB, a DualBeam system dedicated to the preparation of thin, electron-transparent samples from biological specimens. It is said to simplify the cryo-electron tomography workflow by reducing sample preparation time, minimizing the risk of contamination and providing a more precise and detailed view of specific points of interest compared to earlier models.

When used as part of the cryo-ET workflow, the Aquilos 2 allows researchers to prepare more and thinner samples for a clearer view of the inside of cells.

"Sample preparation is manual and can be difficult even for experienced users," says Trisha Rice, vice president and general manager of life sciences at Thermo Fisher. "We’ve improved that in the Aquilos 2 by automating several steps during the process, allowing researchers to focus more on deepening their understanding of a wide range of diseases and speeding the path to drug discovery and treatment."

The Aquilos 2 delivers optimal sample preparation and includes an integrated workflow with dedicated hardware and software that improves productivity and throughput compared to the earlier model.

Coxem introduces new tabletop microscope

The Thermo Scientific Auto Slice and View software, integrated into the Aquilos 2, allows scientists to acquire 3D images in cryogenic conditions by sequential slicing and then capturing multiple images of the interior of a vitrified cell.

The software allows researchers to remove parts of the cell, capture the 3D data from that cell part, and stop capturing data at exactly the right point before moving it to a higher resolution cryo-transmission electron microscope (cryo-TEM).

Additionally, researchers can further increase productivity with the Aquilos 2 by using the Thermo Scientific Cryo AutoTEM software, a guided solution that allows them to mark several points of interest and automatically prepare multiple samples in unattended, even overnight, runs.

Dedicated hardware keeps vitrified samples frozen at all times and protected from contamination.

Finally, the Aquilos 2 includes the Thermo Scientific EasyLift NanoManipulator, which allows researchers to mark several points of interest and automatically prepare multiple samples in unattended, even overnight, runs.

Dedicated hardware keeps vitrified samples frozen at all times and protected from contamination.

Finally, the Aquilos 2 includes the Thermo Scientific EasyLift NanoManipulator, which allows researchers to prepare samples from targeted regions of a cell. Site-specific regions, such as labeled proteins, can be extracted and placed in the inside autogrids for tomogram acquisition in a cryo-TEM.
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Researchers from the National University of Singapore (NUS) have joined an international team of scientists to map an entire human brain at sub-cellular resolution using synchrotron x-ray microscopy. “Our findings could potentially contribute to effective treatment for increasingly important neurodegenerative pathologies such as Alzheimer’s disease and other forms of dementia,” he adds. SYNAPSE will complement its structural data acquisition with the high-speed 100G international network connections of the Singapore Advanced Research and Education Network (SingAREN). The data hub will link all the SYNAPSE partners via the high-speed 100G network to rapidly process, store, mobilise, analyse and access such data.

Biological imaging of live specimens.

“Globally, brain mapping has gained impetus due to the growing impact of such technologies as super-resolution microscopy or cryo-electron tomography,” says Low. “The images captured with unprecedented speed, clarity and granularity by SYNAPSE will form an extensive human brain map.” Our findings could potentially contribute to effective treatment for increasingly important neurodegenerative pathologies such as Alzheimer’s disease and other forms of dementia,” he adds. SYNAPSE will complement its structural data acquisition with the high-speed 100G international network connections of the Singapore Advanced Research and Education Network (SingAREN). The data hub will link all the SYNAPSE partners via the high-speed 100G network to rapidly process, store, mobilise, analyse and access such data.

As mapping a human brain will generate a huge amount of data, a second MOU has been signed by the SYNAPSE members to implement a High Performance Computing network to rapidly process, store, mobilise, access, and analyse such data.

However, which can fit up to three dishes to maximise throughput.

As BRUker points out, the Luxendo TruLive3D Imager system maintains the ease of use and stability of the InVi SPIM and is optimized to allow fast 3D imaging of live specimens. The optical concept, with dual-sided illumination and single-lens detection from below, enables fast acquisition, high-resolution imaging, and minimal shadowing effects, while a wide-field imaging option facilitates sample positioning. The large sample chamber (75 mm in length) can accommodate up to 100 samples into the chamber trough and is ideal for multi-position imaging of small embryos, such as Zebrafish, Drosophila, and mouse, or 3D spheroids.

To image corals, Laissue built a bespoke light-sheet fluorescence microscope to allow gentle observation of the emerging polyps. “Dimming the light has enabled me to show the coral’s dynamics close up, and illustrate the beauty and otherworldliness of these ancient organisms,” says Laissue. “At the same time, we can collect important information about what is happening on the cellular level when corals react to different environmental conditions.”

“This helps us to better understand corals and their development, thus contributing to finding the best strategies to protect and conserve them,” he adds.

Laissue also carries out research at The Marine Biological Laboratory, University of Chicago on local cold water coral, Astrangia poculata, and is continuing development of the light-sheet microscope he used for the winning video here.
Microscopy Analysis
January/February 2020

In situ systems with a difference

Protochips started out as a semiconductor fabrication business but now supplies supports and systems for in situ electron microscopy – why the change? Protochips was founded by myself and David Nackashi in 2002 as a semiconductor fabrication services provider for small companies and academic groups, and through this work, we were introduced to several customers from the electron microscopy market. As electrical engineers with semiconductor experience, we realised that our ability to design and manufacture custom semiconductor products met a growing need for thin-film sample supports with unique properties. Given this, Protochips pivoted from a fabrication services company to a product company focused on providing in situ systems to the electron microscopy market using our semiconductor technology. Since then, we’ve released the Fusion, Poseidon, and Atmosphere product lines and, most recently, the AXON software platform for in situ electron microscopy.

How did you develop the in situ sample supports?
Although myself and David Nackashi are not microscopists, we knew that in situ capabilities like heating, biasing, and confining liquid and gas had to be miniaturised to fit in the TEM. We realised that one way to do that was to integrate these capabilities onto custom micro-electromechanical system (MEMS) chips, known as E-chips. Nanoscale semiconductor films can support a sample and provide a ‘window’ against the vacuum of the microscope while also being thin enough to allow electrons and x-rays to pass right through. Additional processing also turns these windows into heaters – and by stacking two chips we can contain a liquid or gas in a closed cell, creating an environment much more relevant to samples than the high vacuum of a TEM column. In this way we built the platform for a strong business that provides a variety of capabilities to users without the need to customise products.

Have you an example of how E-chips can be used in in situ electron microscopy?
Our Poseidon holder uses pairs of chips with windows to enable basic STEM imaging of samples in liquid. However, using the same holder with a different pair of chips – this time with microfabricated electrodes on and off the window – enables electrochemistry experiments. Also, using the same holder with yet another pair of chips – this time with a heater on the chip – enables studies of samples in heated liquid. These capabilities provide incredible flexibility for researchers.

As well as sample supports, you provide three different in situ cells – what are these?
Poseidon Select is a closed-cell system (using Poseidon E-chips) for imaging samples in native hydrated environments within the TEM. This liquid flow cell can be used to understand relatively unexplored phenomena such as aggregation and self-assembly of nanomaterials, failure mechanisms of lithium ion batteries and the interactions of different biological materials, such as cells with viruses.

Meanwhile, Fusion Select is designed for in situ heating and electrical characterization within the TEM, and is used in material sciences and semiconductor markets. Researchers use the system to understand how, says, chemical composition, crystallinity, and defects change under extreme conditions, including rapid temperature changes up to 1200°C.

Lastly, Atmosphere is a closed-cell system for imaging samples at atomic resolution under different gaseous environments within the TEM. This system targets catalysis markets as well as corrosion and fuel cell markets and can recreate realistic operating environments.

You are also developing sample substrates – Cryo-Chips – for cryo-EM; why?
Cryo-electron microscopy is a growing field and we currently sell a holey carbon grid called ‘C-flat’ as a sample support in this market. Cryo-Chips are a semiconductor replacement for the holey carbon grid. Our prototype has interesting properties that may make it attractive to structural biologists, so we’re evaluating its potential as a new product.

You’ve just launched the AXON software platform for in situ analysis – what is this?
AXON is designed to connect the in situ systems with the microscope and the detector for a seamless workflow that enables researchers to observe material dynamics at a level of detail that we believe was previously impossible. We believe the software is a game-changer in the in situ market – it brings all the focus to the sample and will also make the technique much more accessible to a wider market.

How has the electron microscopy market changed in the last decade? The in situ market has grown significantly over the last decade. More people are using electron microscopy as part of their research work, and more of these people are seeing the strong value that in situ studies can provide.

The market is also shifting towards workflow solutions. Rather than focusing only on resolution or other specifications, users want the entire workflow to drive the highest-quality data. They want sample preparation to be quick, easy, and reliable, and they also want the process of imaging, analysis, and data publication to be efficient. We believe our products help to support these goals. For example, our electrical E-chip for FIB lamella has been designed to convert a risky sample preparation process into one that is easy and reliable, providing quality results in less time. Also, AXON software helps to lower the barriers to using in situ systems by automating tedious tasks such as compensating for sample movement. Such workflow-centric product solutions have followed market demand.

What are your plans for the future? We believe that the in situ market will continue to grow because most people want to see their samples in their natural environment, not the vacuum of the microscope column. Given this, we’re particularly excited about the future for our software, AXON. Workflow solutions that bring together all the major components of the instrument – the microscope, the detectors and the TEM holders – have the power to transform in situ microscopy.

From sample holders to closed cells, Protochips develops in situ systems to make life easier for the electron microscopist. Co-founder and chief technology officer, Dr John Damiano, tells Microscopy and Analysis about his company.

Founding Father John Damiano

Observation of TiO2 overlayer under different conditions using Protochips Atmosphere system Nano Lett. 2016, 16, 4528-4534

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