Simple solutions to advance microscopy imaging

Dr Chandra S Yelleswarapu and **Professor Gopal Rao** discuss their efforts to improve microscope imaging techniques; work that will potentially facilitate important breakthroughs in biomedical research

Can you outline the aims and objectives of your research?

Our group has been involved in basic research in nonlinear optics (like stimulated Raman and Brillouin scattering, self-focusing, saturable and reverse-saturable absorption, etc.) for over four decades. This was mostly publication-orientated, resulting in more than 50 papers in prestigious journals during the last 20 years. As some of these papers involved innovative new concepts, eight patents were granted and two more are underway. The unique feature of our laboratory is that we work on hot topics of current interest using only tabletop experiments without any expensive fancy equipment.

More recently we are looking at translating some of our patents into simple gadgets for the community's benefit, such as laser eye and sensor protection, healthcare and biomedical/biological research particularly relevant to cancer. Right now we are trying to commercialise two of our inventions: firstly, a simple inexpensive gadget for early detection of microcalcifications in mammograms for cancer diagnostics; and secondly, an optical Fourier phase contrast microscope (FPCM) which offers several advantages over those currently available. We also have an associated technique displaying phase and fluorescence at the same time which is useful for cell biologists. We believe this will facilitate significant breakthroughs in biological and biomedical research areas.

Can you offer some insights into the basic mechanisms by which your FCPM technique operates?

Firstly, the technique involves picking a laser light source to facilitate optical Fourier processing where the object information is spatially mapped at the Fourier plane (focal plane of the converging lens), called the Fourier spectrum. The Fourier spectrum of an object contains low spatial frequencies at the centre with high intensities while high spatial frequencies are on the edges with low intensities. This enables us to selectively alter the information so that the phase object is visible to the naked eye or a camera. Secondly, a self-adaptive all-optical liquid crystal phase filter has been developed. When the phase object information is transformed onto the liquid crystal cell, owing to high intensity of low spatial frequencies, only the liquid crystal molecules situated at the centre of the Fourier spectrum transform selectively into the isotropic phase. On the other hand, high spatial frequencies on the edges of the Fourier spectrum are weak enough to not induce any phase transition in the liquid crystal and hence are in anisotropic phase. Thus the low spatial frequencies transmitting through the self-induced isotropic phase of the liquid crystal cell is unaltered while the phase of the high spatial frequencies transmitting through the liquid crystal cell acquires additional phase. As a result, there is a $\pi/2$ phase difference between high and low spatial frequencies which is the fundamental requirement for phase contrast imaging.

To what extent does this constitute a major breakthrough in the development of cutting-edge microscope technology?

By incorporating a notch filter the FPCM functions as a common-path multimodal microscope. A notch filter blocks a narrow band of light frequencies and in the present case it is used to block the excitation laser light. Cell response to the environment is complex, involving both structural and functional changes. Understanding of this response is sometimes limited by the ability to image cell structure and function at the same time. Cell biologists currently obtain separate images, such as phase and fluorescence features, using two or more different imaging techniques one after the other and digitally register these images together. Using our multimodal microscope we obtain a real-time display of phase (or absorption) and fluorescence features at the same time to study live cell dynamics. One can record the sequence of fast processes in a movie format. Further, spatial frequency filtering enables the display of shape and size of live cells. In principle, wherever the current phase contrast microscope is used, the FPCM will do a better job. The FPCM will be especially useful for biological and biomedical research, applications for basic research in cell biology, and cancer research.

How has the interdisciplinary approach adopted during the project been important to its success?

As physicists we developed the technique but we need active collaboration with biologists to test the performance of the system and compare with the current state-of-the-art commercial microscope. We also need a variety of samples to check the versatility of the instrument. We were able to obtain spectacular pictures of *Drosophila* embryos, provided by our biology colleague Professor Alexey Veraksa. We recorded an interesting movie of amoeba, provided by our biology colleagues and constructive feedback from our collaborators helped us fine-tune the system.

Redefining traditional biological microscopy imaging

A team based at **University of Massachusetts Boston** has developed novel Fourier phase contrast microscope instruments, building a solid platform for future biomedical and biological research

TRADITIONAL MEDICINE AND biology have always been challenged by not being able to see transparent objects with just the human eye, or even a camera, because there is no absorption of the visible light. This has meant that biological and biomedical research scientists have to stain samples for viewing. The development of the phase contrast microscope in the early 1930s meant that living cells could be examined in their natural state without being fixed or stained. Phase contrast microscopy imaging has since become an important analytical tool in many laboratories and is now a standard methodology used in a number of medical fields, such as pharmacology, neurobiology and developmental biology.

The traditional commercial phase contrast microscopy instruments use tungsten-halogen lamps as the light sources, a condenser annulus for separating the deviated and undeviated light and a phase plate for generating the required additional phase retardation. These plates (both condenser annulus and phase plate) are matched in diameter and optically conjugate to each other. Over the years, while the technological advances in terms of phase plate design, detection and display schemes have been incorporated into microscopy imaging instruments, the source has remained the same.

Led by Professors Chandra S Yelleswarapu and Gopal Rao, researchers at University of Massachusetts Boston have developed a Fourier phase contrast microscopy (FPCM) imaging technique which offers several unique advantages over the traditional methods. The FPCM concept exploits monochromaticity and phase coherence characteristics of a low power laser. Monochromaticity of the laser source provides precisely defined deviated light and also delivers well-resolved Fourier plane mapping of spatial frequencies (object information). The intensity of the laser source means that features are bright and clearly visible. A high degree of phase coherence preserves the phase retardation introduced by the liquid crystal. The FPCM principle also involves the generation of required additional π/2 phase retardation between the deviated (diffracted) and undeviated wavefronts emerging from the specimen in addition to the π/2 already present (an assumption for small phase differences). When the liquid crystal cell is placed at the Fourier plane, low spatial frequencies at the centre are intense enough to induce local liquid crystal molecules into an isotropic phase whereas high spatial frequencies on the edges are not so intense and remain in the anisotropic phase, resulting in π/2 phase difference between high and low spatial frequencies in real time. Interference between the two beams facilitates the amplitude contrast resulting in image acquisition.

USING A SIMPLE MULTIMODAL IMAGING SYSTEM

Fluorescence imaging enables tracking of specific molecules inside the cell and organelles in cellular transport, cellular communication and physiological events. Often, additional fluorescence labelling is used to observe the cellular or tissue structures. This labelling process can be avoided by imaging these structures using

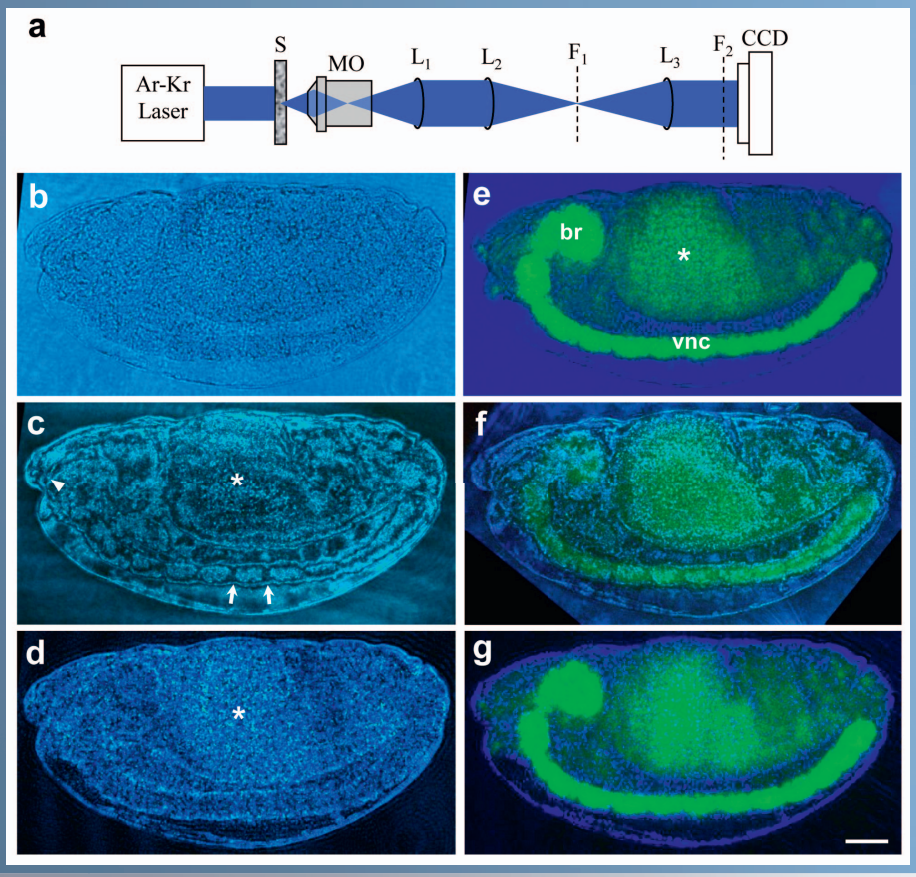


FIGURE 1. CMOM System and its application in imaging amplitude, phase and fluorescence features of a *Drosophlia* embryo. All panels show the same embryo, stained with anti-elav primary antibody to visualise the nervous system, followed by FITC-conjugated secondary antibody. Scale bar. 50 µm. **a)** Schematic of the experimental setup. S, sample; MO, microscope objective; L, lens; F_1, Fourier plane, F_2, filter plane. **b)** Conventional bright field image. Internal structures of the embryo are not well defined. **c)** FPCM image obtained using a phase filter at F_1 and neutral density filter (NDF) at F_2. Segmental boundaries are clearly visible in this sagittal image (arrows), as well as the invaginating foregut primordia at the stomodeum (arrowhead), the midgut area (asterisk), and other internal features. **d)** Edge-enhanced image obtained with a high-pass spatial filter at F_1 and NDF at F_2. Inner yolk containing cells are optically dense and appear as a lighter area in the middle of the embryo (asterisk). **e)** Fluorescence image with laser line notch filter (NF) at F_2 and no filter at F_1. The segmented ventral nerve cord (vnc) and the brain region (br) are detected in the sagittal optical section of the embryo. Although the NF blocks most of the excitation source, a small amount of light leaks through, making it possible to observe the overall embryo morphology. The midgut is autofluorescent (asterisk). **f)** Phase+fluorescence image obtained with a phase filter at F_1 and NF at F_2. Both the FPCM and trans-fluorescence information are recorded at the same time. Most of the information visible in (c) is retained, with the nervous system and midgut fluorescence also detectable. **g)** Edge enhanced+fluorescence image obtained with a spatial filter at F_1 and NF at F_2. The boundary of the embryo is better resolved (compare to (e)), and the fluorescence signal is clearly visible.

a phase contrast microscope. However, the current technology cannot provide both phase and fluorescence images at the same time. So cell biologists obtain separate images, such as phase and fluorescence features of biological specimens, using two or more different imaging techniques, one after the other, and then digitally combining them.

The commercial phase contrast microscopy imaging procedure cannot meet the needs of biologists in simultaneous real time monitoring of phase and fluorescence features that occur on short timescales of the order of micro- or even nano-seconds. This implies that the traditional microscopy imaging techniques have a number of drawbacks that are impacting on the capacity of scientific investigations to move forward. Yelleswarapu and Rao's team are looking at opportunities to fill this instrumentation gap. Indeed, they explain that simply by adding a notch filter, the FPCM functions as a common-path multimodal microscope, providing real time display of phase and fluorescence features at the same time. The notch filter simply blocks a narrow band of light frequencies, such as the excitation laser light.

The research team has set up experiments to image the phase and fluorescence features of a *Drosophila* embryo. As would be expected, the internal features of the embryo were not clearly visible in the brightfield image obtained using an ordinary microscope and so a high contrast FPCM image was then obtained. This was done by placing liquid crystal cell at the Fourier plane, and as a result, several distinct features of the *Drosophila* embryo such as segmental boundaries (arrows), the anterior mouth opening, or stomodeum (arrowhead), the midgut (asterisk), as well as other internal structures were clearly visible.

Yelleswarapu and Rao explain that this relatively simple multimodal imaging system has the ability to record all the features of a biological specimen without switching optical paths. This means there is no longer a need to acquire separate images followed by image registration and the digital merging of these on a computer. The research team are pleased with their results and they have even recorded a movie for an amoeba sample displaying the movement of the nucleus, contractile vacuole and smaller organelles that move within the cytoplasm. "As a consequence," explains Rao, "the FPCM technique has the potential to help deliver significant breakthroughs in biological and biomedical research."

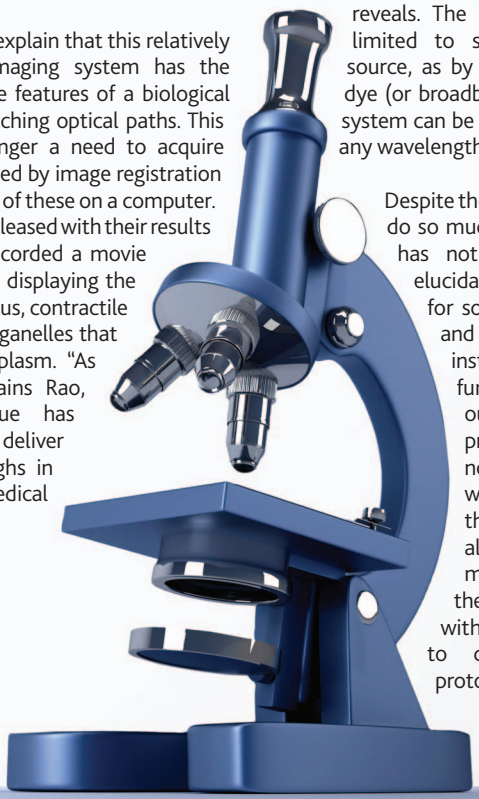
THE CHALLENGE OF INTENSITIES AND FREQUENCIES

The FPCM instrument is considered to be far more user-friendly and delivers improved reliability than the traditional models. A range of additional benefits have been gained through the advancements the research team have produced. Phase halos are eliminated with increased image contrast; FPCM does not require any moving parts such as the condenser annulus-phase plate, and the system is self-adaptive to changes in shape and size of phase objects. "The FPCM functions as an ordinary bright-field microscope at low intensities of the source and just by increasing the intensity it converts to a phase contrast microscope," points out Rao. He details how the instrument offers the additional advantage of displaying shape and size of cells as spatial filtering is involved – a technique particularly useful for cell biologists interested in morphology.

The focus of the Fourier lens has low spatial frequencies at the centre with high intensities and high spatial frequencies on the edges with low intensities. This has created a number of challenges for the project team. It was important that the team picked an appropriate room temperature liquid crystal – which remains anisotropic on the edges as well as going into isotropic phase at the centre with a very small change in temperature.

SUCCESS IN SIMPLICITY

Yelleswarapu describes how the FPCM technique is both self-adaptive and user-friendly. He observes that no alignment is necessary when there is a change in shape, size and magnitude of phase variations of phase objects; thus, everything can be controlled by changing just one parameter. "No moving parts and no requirement for alignment of condenser annuls and phase plate brings robustness," he reveals. The FPCM technique is not limited to single wavelength light source, as by the addition of suitable dye (or broadband absorbing dyes) the system can be used with light source of any wavelength of interest.



Despite their progress, being able to do so much with so few resources has not come easily, as Rao elucidates: "It is an uphill battle for some of us at less known and not so well-resourced institutions to get research funds and also to publish our research results in prestigious journals". But now the research team are well on the way to realising their vision. They have already built a laboratory model and they are in the stage of negotiating with several companies to develop an industrial prototype for manufacturing the instrument and then marketing it.

INTELLIGENCE

FOURIER PHASE CONTRAST MICROSCOPY TECHNIQUE FOR BIOMEDICAL RESEARCH

OBJECTIVES

The team developed a novel Fourier phase contrast microscopy (FPCM) imaging technique exploiting monochromaticity and phase coherence characteristics of a low power cw laser as the source and photo-induced birefringence of a nematic liquid crystal for introducing the additional π/2 phase difference required between deviated and undeviated beams.

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