

Light field microscopy: principles and applications

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Introduction

The past two decades have seen huge developments in optical, and particularly fluorescence, microscopy. Techniques from super-resolution to high-throughput imaging provide scientists with an armoury of different methods with which to visualise structures and processes with unprecedented

spatio-temporal resolution. Fundamental to the differences between these methods, and their relative strengths and weaknesses, is the dimensional way in which image data is captured as illustrated in Figure 1. Point-scanning techniques, such as confocal microscopy, offer high spatial resolution at the expense of slow image capture speed and

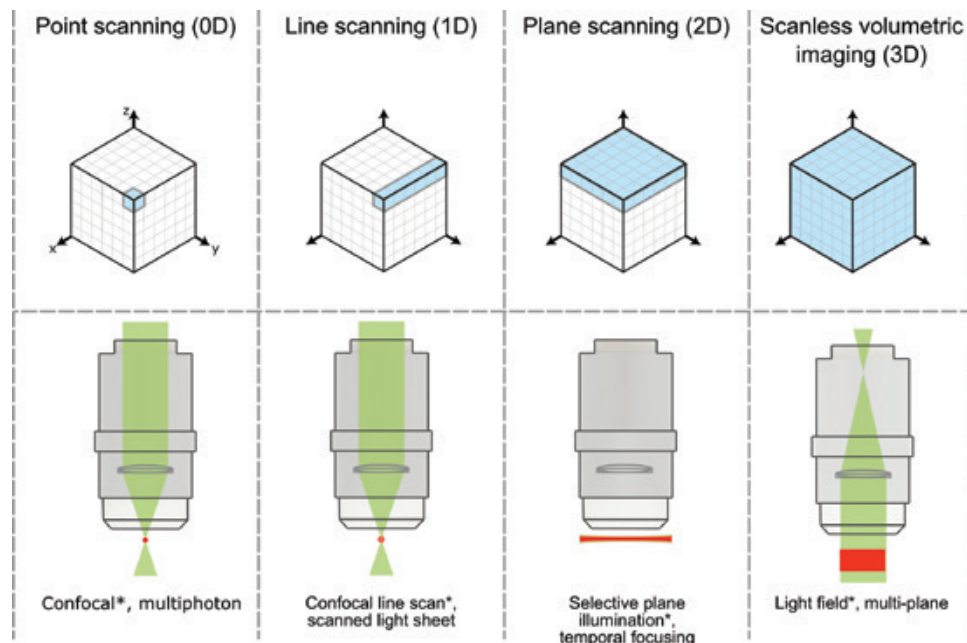


Figure 1. Different approaches to 3D microscopic imaging. (top, from left) point scanning techniques build up an image by raster scanning focused illumination through the sample. Line and plane scanning methods parallelise this to increase imaging speed. Scanless volumetric imaging methods are capable of capturing 3D image information in a single camera exposure. Examples of each technique, with illumination and effective imaging volume within sample shown in green and red respectively for the method marked with *.

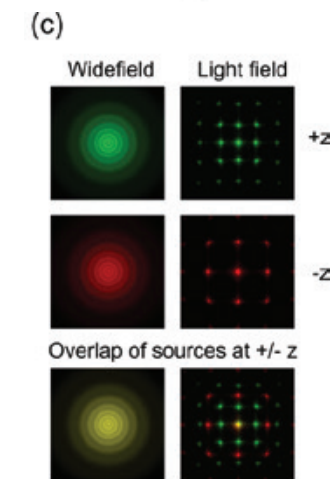
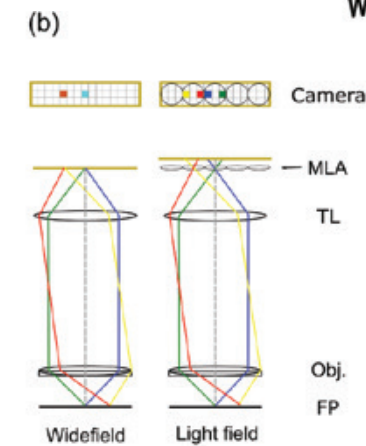
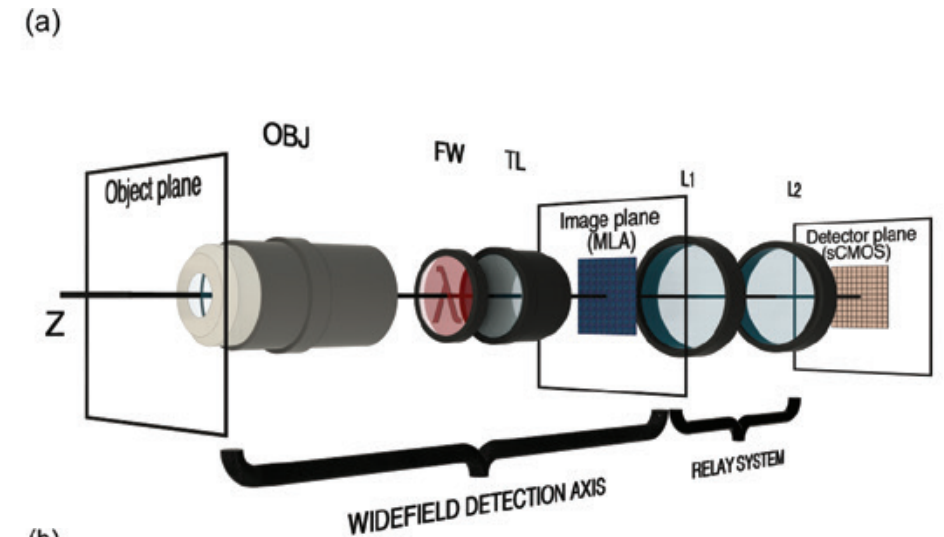


Figure 2. Schematic diagram illustrating the principles of light field microscopy. (a) Schematic diagram showing the principal components in a light field microscope. OBJ – objective lens, FW – filter wheel, TL – tube lens, MLA – microlens array, L1 and L2 – relay lenses. (b) In a conventional widefield microscope (left) all rays emerging from a point in the object, which are collected by the objective lens, are focused to the same point on the camera sensor and information about the direction of propagation of light rays is lost. In a light field microscope (right) an MLA mounted at the native image plane directs rays propagating in different directions on to different camera pixels, allowing reconstruction of perspective views of the object. (c) Simulated images of two point emitters axially offset by the same distance (z) either side of the focal plane shown in red and green. In a conventional widefield microscope (left) the irradiance pattern from both points overlaps at the image plane. In a light field microscope (right) the same points give rise to distinct non-overlapping patterns on the camera which can be analysed to determine their axial position and digitally refocus the image after capture.

spatial resolution. However, all these techniques are inherently sequential, requiring a 3D image to be built up from a series of individual images. The necessity of scanning fundamentally limits temporal resolution and leads to increased photobleaching and phototoxicity in biological specimens. By contrast, scanless volumetric imaging techniques illuminate the *entire specimen* and collect light from *every point* in the volume of interest simultaneously, allowing 3D imaging at a speed limited only by the frame rate of the camera. This article describes one such volumetric imaging technique, Light Field Microscopy (LFM), in which a microlens array is used to capture both spatial and angular information about the sample simultaneously. We describe the basic principles, advantages and limitations of LFM and discuss recent applications of this fast 3D imaging technique.

high sample light-dose. Increasing parallelisation offers improved spatial resolution and a reduction in sample light dose, typically at the expense of

Principles of light field microscopy

In photography and computer vision the term light field is used to describe the distribution of radiance from an object [1], or the amount of light emitted by the object as a function of both position and view direction. In a conventional widefield microscope, all rays emanating from a point in the object within the acceptance cone of the objective lens (determined by its numerical aperture) are focused to a common point on the camera sensor and directional (angular) information is lost. LFM techniques seek to capture 4D radiance information simultaneously, providing volumetric information about the object at a speed limited only by the frame rate of the camera.

Although related to the idea of integral photography proposed by Gabriel Lippmann in the early 20th century [2] and more recently developed for photographic applications [1, 3], the principles of LFM were first described by Marc Levoy and colleagues at Stanford University [4]. The method relies on a simple modification to a conventional widefield microscope system, whereby a microlens array (MLA) is mounted in the native image plane and the camera is shifted to the back focal plane of the MLA (Figure 2(a)). A raw LFM image is a 4D dataset containing information about the radiance of light emanating from the object as a function of spatial location in the sample (x, y) and propagation direction (u, v) and consists of an array of circular subimages. Each subimage represents a different location in the sample and each pixel within a subimage corresponds to light emanating from that location in a particular direction. The concept is illustrated in Figure 2(b) which shows rays propagating in different directions from two different points in the focal plane of a microscope objective. In a conventional microscope (left) the two rays from each point are brought to a common focus at the camera, represented as a pair of (purple) image pixels. In a light field microscope (right) the MLA separates rays propagating in different directions on to different camera pixels. Subsampling the LFM

image by taking the same pixel from each subimage gives a view of the object from a particular direction. Each of these perspective views uses only a fraction of the full aperture of the objective lens, the depth of field is significantly increased when compared to a conventional microscope image [4].

Summing the pixels within each microlens subimage results in a conventional image of the object focused at the native object plane (the front focal plane of the detection objective). However, the directional information also enables us to compute what the raw light field image would look like if the camera were axially displaced. In practice, this amounts to shearing the four dimensional light field, equivalent to shifting each perspective view before summing pixels under each microlens, or taking a rotated 2D slice of the light field's Fourier transform and performing an inverse Fourier transform [5]. This digital operation, which can be performed in real-time using a standard computer, allows post-capture focusing and computation of a focal series (z-stack) from a single camera exposure, making light field microscopy well-suited to imaging dynamic 3D samples. Conventional iterative 3D deconvolution can be applied to the focal stack to reduce out-of-focus blur in each image slice [4].

The cost of this additional directional information is a significant reduction in lateral spatial resolution. In the classic light field microscopy configuration (Figure 2(a)) spatial sampling of the image is determined by the pitch of the microlenses in the MLA, which is significantly larger than the camera pixels. As a result the image is undersampled, leading to a loss of spatial information and potential introduction of aliasing artefacts. There are several methods for improving spatial resolution without sacrificing field of view (by increasing the image magnification) or reducing angular sampling and depth of field by reducing the MLA pitch. The spatial sampling rate can be increased by capturing a series of images as the MLA is shifted laterally [6], however this comes at the expense of a longer total image acquisition time. Although the native

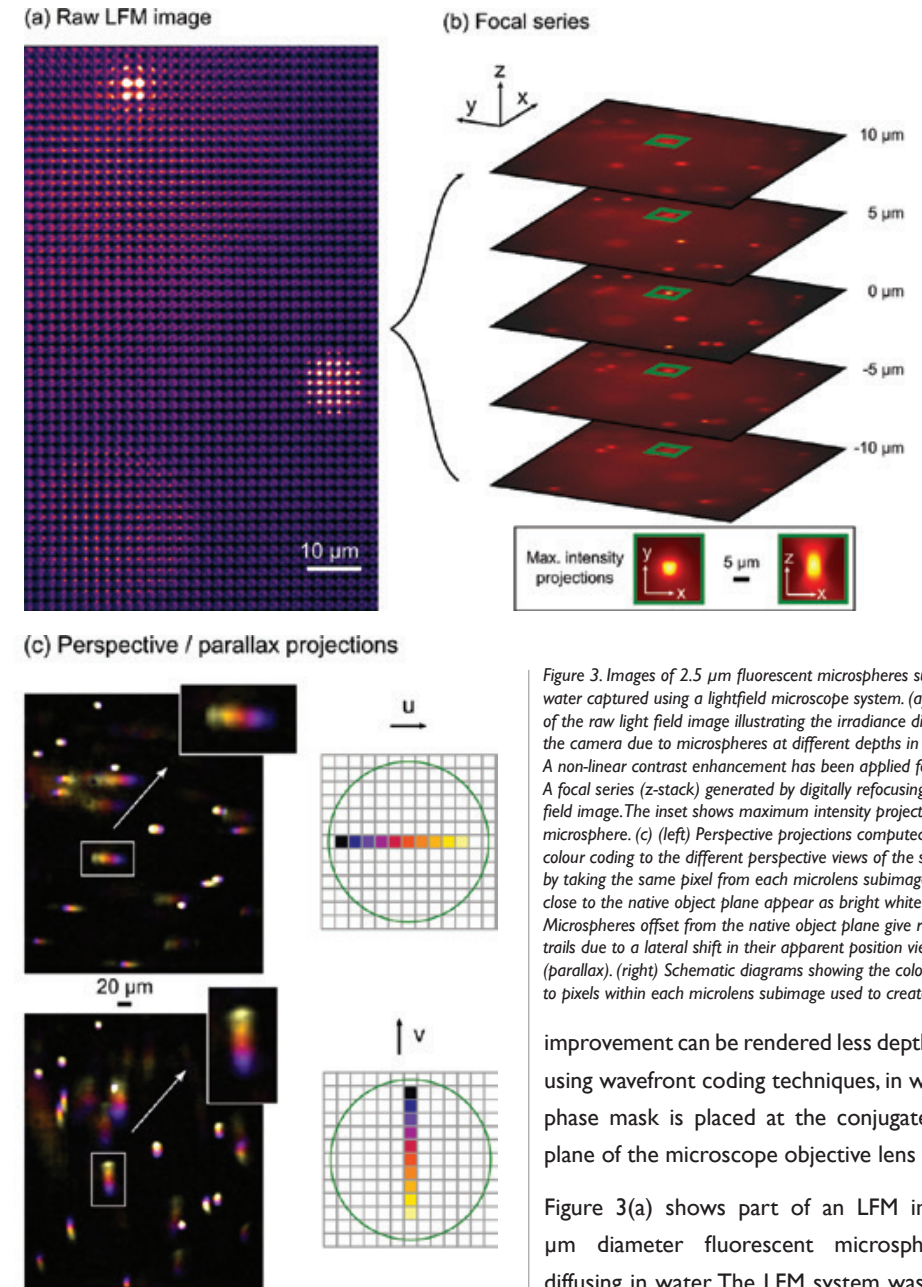


Figure 3. Images of 2.5 μm fluorescent microspheres suspended in water captured using a lightfield microscope system. (a) Cropped region of the raw light field image illustrating the irradiance distribution at the camera due to microspheres at different depths in the sample. A non-linear contrast enhancement has been applied for clarity. (b) A focal series (z-stack) generated by digitally refocusing the raw light field image. The inset shows maximum intensity projections of a single microsphere. (c) (left) Perspective projections computed by applying colour coding to the different perspective views of a single microsphere. Microspheres close to the native object plane appear as bright white spots. Microspheres offset from the native object plane give rise to coloured trails due to a lateral shift in their apparent position view direction (parallax). (right) Schematic diagrams showing the colour coding applied to pixels within each microlens subimage used to create the projection.

improvement can be rendered less depth dependent using wavefront coding techniques, in which a cubic phase mask is placed at the conjugate back focal plane of the microscope objective lens [8].

Figure 3(a) shows part of an LFM image of 2.5 μm diameter fluorescent microspheres freely diffusing in water. The LFM system was based on a standard upright widefield microscope (Olympus BX51 with LUMPLFLN 60XW objective lens) with a rectangular array of microlenses (125 μm pitch, $f/30$) mounted at the native image plane. An image of the back focal plane of the MLA was projected on to the camera sensor using a macro relay lens [9]. Information about the depth of each microsphere within the sample is encoded in the corresponding brightness distribution in the camera

object plane is sparsely sampled in an LFM system, tracing ray trajectories back through the object reveals that sampling is increased for out-of-focus parts of the object. This observation, combined with knowledge of the full light field point spread function (PSF), allows for the recovery of higher resolution information by reconstructing images using 3D deconvolution [7]. Further, this resolution

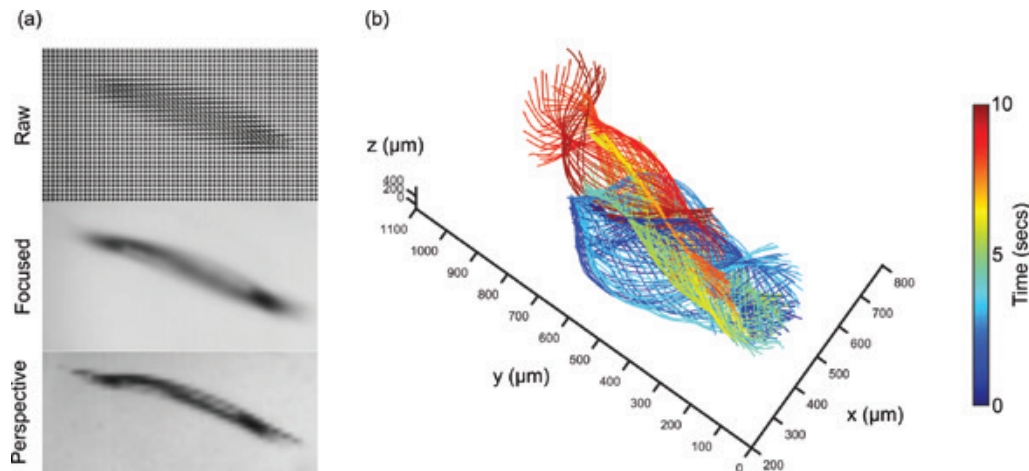


Figure 4. 3D behavioural phenotyping of *C. elegans* using light field microscopy. (a) Example raw light field image (top), focused view (middle) and perspective view (bottom) of a wild type adult stage *C. elegans* specimen in an agarose gel. (b) 3D midline skeletons extracted from a timelapse light field image sequence of the moving organism.

image. Figure 3(b) illustrates how digital refocusing allows individual microspheres, at different depths, to be brought into focus. The inset shows xy and xz maximum intensity projections of a single microsphere with a corresponding full-width at half maxima of 3.8 μm and 6.2 μm , respectively. Figure 3(c) represents the directional information contained within the light field image by applying a colour coding to the perspective views formed from single pixel perspective views horizontally (u , top) and vertically (v , bottom) through the centre of each microlens subimage. Due to the apparent lateral shift in their position with view direction (parallax), microspheres offset from the focal plane of the objective lens give rise to colour trails in the projection image. Microspheres close to the native appear as bright white spots.

Applications

All microscopic imaging techniques represent a compromise between different performance characteristics including: spatial resolution (lateral and axial); image capture speed; illumination intensity; sensitivity; field of view and depth penetration. LFM excels at fast, volumetric imaging with moderate spatial resolution and for this reason the technique has often been applied to capture dynamic events

in 3D samples. Several research groups have used LFM to measure neural activity in model organisms expressing genetically encoded calcium indicators (GECIs) [10-12], where simultaneous recording of the fluorescent signal within an extended volume allows for accurate measurement and timing of the response of individual neurons. For similar reasons, LFM has also been used in 3D particle velocimetry experiments for the experimental determination of fluidic flows [13]. Polarised light field microscopy, in which an MLA is incorporated into a polarised light microscope, has been used to measure the birefringence of anisotropic materials such as calcite [14, 15]. Another non-biological application for which LFM has been used is to investigate the properties of clouds of fluorescing atoms in a magneto optical trap [16].

In the Touch Lab at UCL, we have used LFM to study the microscopic roundworm *C. elegans* and, in particular, to explore the relationship between organism biomechanics and mechanosensation [17]. Using LFM we were able capture the response of touch receptor neurons (TRNs) expressing a GECI (GCaMP6s) during controlled mechanical stimulation of the organism using a microforce sensing probe [9]. These data allowed us to explore touch sensitivity empirically and, importantly,

capture simultaneous responses from different TRNs located at different depths within the organism. More recently, we have used light field microscopy for time-lapse imaging of *C. elegans* specimens moving freely in 3D environments such as agar and aqueous buffer solution. The limitations of conventional 3D microscopic imaging techniques mean that such behavioural phenotyping is usually limited to studying 2D posture and motion on planar substrates, which fail to replicate the freedom and complexity of *C. elegans* native environment. By extracting specimen depth information using a computational depth estimation technique which combines depth cues from defocus and parallax (correspondence) [18], we were able to extract a 3D midline skeleton for the organism from each light field image (Figure 4(b)). This midline skeleton was then further analysed to compute metrics for posture and motion to compare the behaviour of cuticle collagen mutants quantitatively and to study the swimming motion of the organism [19].

Outlook

LFM's capacity for capturing volumetric information in a single camera exposure makes it ideal for imaging fast-changing, dynamic systems with moderate spatial resolution. However, the need to sample and record (4D) spatial and directional information on the same camera sensor inherently limits lateral spatial resolution and field of view. Recent work has demonstrated that using a more complex MLA placed conjugate to the pupil of the objective lens (rather than the native image plane) can enhance spatial resolution while preserving working volume [10]. Spatial sampling and spatial resolution can also be increased using an array of cameras, each capturing a view of the sample along a different direction [20]. The depth-of-field of an LFM system can be increased by using a beamsplitter to separate the emitted light into multiple channels each containing a camera and MLA focussed at a different depth in the sample [21]. New image reconstruction and analysis approaches can also mitigate the problems of low

spatial resolution and light scattering inherent in conventional LFM. For example, by exploiting spatial and temporal sparsity in time-lapse LFM images, compressive sensing techniques have been used to extract information from raw light field images without needing to reconstruct a 3D volume image [22]. This approach has been shown to allow robust detection and monitoring of individual neurons in the presence of imaging aberrations and scattered light, enabling measurements of activity over large networks of neurons. Further developments in hardware design, camera and MLA technology and image reconstruction and analysis methods offer the possibility of further improving LFM imaging performance, enabling broader application of LFM techniques in biological imaging and beyond.

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