Overview of the technology and product innovation

Overview of advanced 3D light microscopy[1]

Introduction to microscopy

1. There are three main, fundamentally different, types of microscopy:

   - light microscopy;
   - electron microscopy; and
   - scanning probe microscopy.

2. Light microscopy is based on long-established principles and is the technology used in basic microscopes such as those commonly used in schools. Light microscopes use optical methods to create an image from the light that is absorbed by, reflected[2] by or emitted from a specimen that is being examined, or whose propagation is retarded differentially by a specimen.

3. Electron microscopy uses a beam of electrons to examine objects. The systems are analogous to light microscopy but can obtain far higher resolutions as the wavelength of electrons can be far shorter than that of visible light. Samples are examined in partially or wholly evacuated chambers and normally require harsh methods of preparation (for example, coating with metals) restricting the range of applications.

4. Scanning probe microscopy covers several related technologies for imaging and measuring surfaces on a fine scale, down to the level of molecules and groups of atoms. The general approach is to scan an extremely sharp tip across the object surface. The three most common scanning probe techniques are: *atomic force microscopy,* which measures the interaction force between the tip and surface; *scanning tunnelling microscopy,* which measures a weak electrical current flowing between tip and sample; and *near-field scanning optical microscopy,* which scans a very small light source very close to the sample.

Light microscopy

5. Basic light microscopes utilize light reflected by or absorbed by specimens to make them visible. The examination of reflected and absorbed light, together with light whose propagation has been retarded differentially, is rarely capable of depicting small amounts of diverse but tightly defined substances. To examine these, specimens are stained with one or more highly specific markers which can be made to fluoresce. Light of one colour is absorbed (a process known as *excitation*) and then most of this energy is re-emitted as light of a different, almost invariably red-shifted, colour (a process known as *fluorescent emission*). Fluorescence microscopy

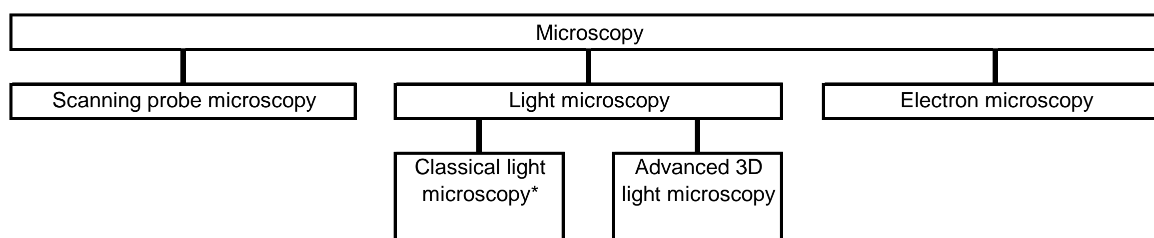
[1]The CC engaged Dr Alan Entwistle of the Ludwig Institute for Cancer Research as a technical adviser and gratefully acknowledges his contribution to the inquiry and in particular to this section of the report. The CC is responsible for this report and the conclusions therein.

[2]'Scattered' is technically a more correct term than 'reflected', however the latter is widely used.

possesses fine sensitivity. Fluorescence techniques underlie the following discussion, but the other techniques are still very important, in a very wide variety of specific applications.

6. Light microscopy can be divided between classical light microscopy and advanced 3D light microscopy. This broad classification is illustrated in Figure 1.

FIGURE 1
Classification of microscopy



Source: CC.
*Also known as wide-field microscopy.

7. Advanced 3D light microscopy refers to a range of techniques that enhance the abilities of the classical light microscope to create 3D[3] images where resolutions as small as 0.2 micron can be obtained, at least in theory. Specifically, 3D light microscopes greatly improve the resolution that can be perceived in the axial direction, ie the resolution of different objects that come in and out of view as the focus control of the microscope is adjusted.

8. 3D light microscopes are typically used for the examination of semi-conductors and in biological and materials science research. Their use is especially important in the fields of cell biology, cell motility, developmental biology and neurobiology. Newer technologies may give rise to medical uses.

9. In addition to the technologies under review, materials scientists who examine opaque specimens also often use interferometric methods based upon the differential retardation of the propagation of light.

Types of advanced 3D light microscopy

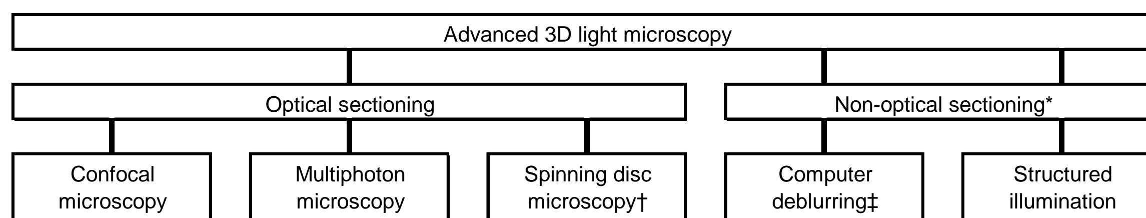
10. To obtain 3D images the microscope is used to obtain data from numerous points across and within the specimen. Thus images can be built up based on a series of pixels, each of which has three coordinates (x, y, z), where, conventionally, the x and y coordinates describe lateral position and the z-coordinate describes the axial position, ie the position along the line of focus. Gathering data from all these points requires sophisticated optics, mechanics and software to control the microscope system. It can also be time-consuming. Some systems obtain the data point by point, others scan multiple individual points concurrently and some work by processing data collected simultaneously over the complete lateral matrix.

[3] In this report we use the term 3D exclusively to refer to the spatial dimensions, however microscopists also include temporal and other measurements as dimensions in their terminology.

11. 3D light microscopy has to separate light that emanates from a chosen plane (z-coordinate) in the specimen from light that arises from below and above that plane.

12. There are two approaches to 3D light microscopy, for which we have used the terms optical and non-optical sectioning.[4] The first of these uses lenses and baffles to select in-focus data for the 3D images—although computers are often needed to assemble and view the images. The second approach uses mixtures of optical methods and sophisticated data processing to produce optical sections. Each of these two approaches includes different techniques. A broad classification of advanced 3D light microscopy is provided in Figure 2.

FIGURE 2
Classification of advanced 3D light microscopy



Source: CC.
*The term non-optical sectioning does not mean that optics are not used in these systems; Bio-Rad prefer the term 'computer processed microscopy'.
†We also include in this category similar 'tandem scanning systems'.
‡Computer deblurring can be used with wide-field microscopes or with laser scanning systems.

(a) **Confocal microscopes** focus light, from one or more lasers, down to the smallest possible spot, collect the light that emanates from that spot and focus it on to a pinhole. This light passes through the pinhole into the detector whereas light that originates from progressively distant zones from the region of focus is increasingly rejected by the walls around the pinhole and fails to enter the detector. Hence, the light originating from the preferentially illuminated spot is also sampled preferentially by the detector. To build up a 2D image the specimen is scanned line by line in a raster fashion, similar to that used for a conventional television screen. A 3D image is built up by collecting a series of these 2D scans at different focal positions. The term **confocal laser scanning microscopy** (**CLSM**) is often used for these systems.

(b) **Multiphoton microscopes** use lasers that produce very brief, high-intensity pulses of infrared light. In the spot at the focus of a microscope the light is so intense that fluorescent molecules can be excited by two or more closely spaced infrared photons instead of the usual single visible or ultraviolet photon. As the necessary intensities of light are only obtained within and immediately around the spot at the focus they avoid the creation of undesired fluorescence elsewhere; this contrasts with the confocal techniques where fluorescence is induced at all depths (z-coordinates) of the specimen and the out-of-focus information is subsequently optically removed. Multiphoton systems are almost all based on the point by point laser scanning approach described above.

[4]Both technologies use optics to function. Bio-Rad prefers a classification that uses the terms 'laser scanning microscopy' and 'computer-processed microscopy'.

(i) Multiphoton microscopes do not actually require a detector pinhole, unlike confocal microscopes. However, since they are usually based on upgrades of confocal systems, most multiphoton systems do include a detector pinhole and can also be used as confocal systems.

(ii) The principal advantage of multiphoton systems is that they enable significantly deeper penetration into the specimen. In specific experiments it has been found that they cause less damage to the specimen, but these observations cannot be generalized and are more likely to be the exception than the rule.

(iii) Multiphoton systems tend to be classified as using subpicosecond[5] (less than 10^{-12} second) or picosecond (greater than 10^{-12}) technology. The technologies are not essentially different; the terminology describes the duration of the infrared laser pulse, and the distinction has largely arisen due to patent issues. The pulse duration is an important factor in the performance of these microscopes but the optimal duration of the pulse for any specific use has yet to be agreed upon.

(c) **Spinning disc or tandem scanning microscopes** employ a rotating disc with very many (typically in the order of 10,000) holes to acquire data from many points concurrently. Each hole behaves like an individual confocal microscope and, because so many points are scanned simultaneously, these systems offer advantages when it is essential to acquire data as rapidly as possible. Initially these instruments used the illumination very inefficiently but by collecting the illumination with an array of tiny lenses that focuses it upon the holes this limitation has been overcome.

(d) **Computer deblurring or deconvolution** entails taking a succession of differentially focused wide-field images and extracting the in-focus information from them by comparing the wide-field information with descriptions of the blurring expected. Several mathematical models are used to generate the descriptions of blurring and consequently a variety of algorithms can be used to process the image data. Although presented here as an alternative to optical sectioning, these techniques can also be used in conjunction with optical sectioning.

(e) **Structured illumination methods** involve the introduction of a patterned mask into the optical system and the acquisition of two, or more, images created with the mask in different positions. A computer then combines these multiple images in a manner that eliminates the blur, leaving just the in-focus information behind.

13. The different systems have advantages and disadvantages. Cost is the most obvious—we were told that to install a multiphoton system costs from around £75,000 more than a laser scanning confocal system, most of the difference being explained by the cost of the pulsed laser. We were told that spinning disc systems and the non-optical sectioning systems can be significantly less expensive than confocal systems. For each type of system there is a wide range of products and options available such that there are broad bands of prices and overlaps between the costs of various types of system. For example, some confocal systems cost more

[5]Subpicosecond systems are also referred to as femtosecond systems.

than some multiphoton systems and there is price overlap between some non-optical sectioning systems and some confocal systems.

14. Although they tend not to give the best resolution, we were told that spinning disc systems and the non-optical sectioning systems can have advantages in addition to generally lower costs. In particular, speed of data acquisition is often fast which is important for certain types of experiment.

15. Key factors, in addition to cost of purchase, in assessing a 3D light microscope include:

- sensitivity of detection;
- speed of data acquisition;
- phototoxicity (specimen damage due to light exposure);
- processing time;
- features that extend the range of experiments that can be carried out; and
- running and maintenance costs.

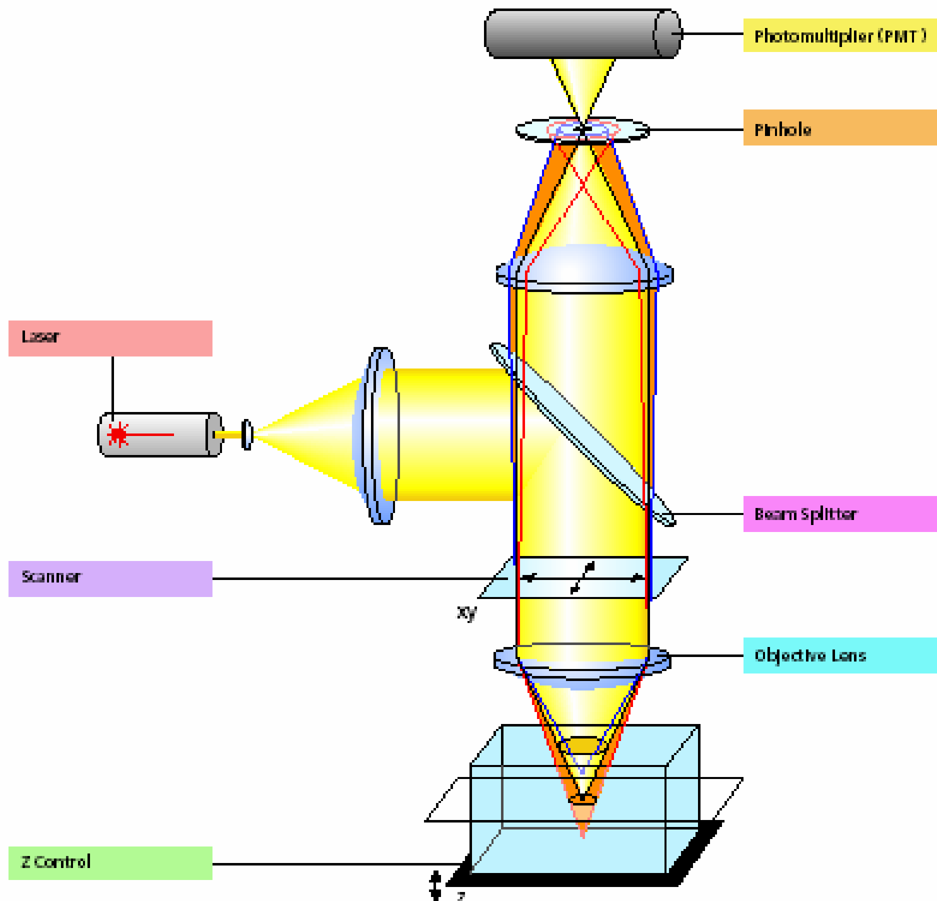
16. Multiphoton systems are expensive and are a more recent technological development than confocal systems. The main benefit of multiphoton microscopy is that the use of infrared illumination allows structures that lie deeper within specimens to be observed more clearly. In addition, when multiphoton illumination is used instead of ultraviolet illumination the specimen will normally undergo less photo-damage. The two systems have, however, such different profiles of strengths and weaknesses for different types of work that they are usually complementary methods.

Features of confocal and multiphoton systems

17. Figure 3 illustrates the general design of a confocal laser scanning microscope. In general the equipment appears as a normal microscope (with eyepiece) with a series of attachments:

- Laser equipment to excite fluorescence. The pulsed lasers used for multiphoton systems are very large although they are less bulky than they used to be. The lasers used for confocal systems are very much smaller.
- Optic fibres to connect the laser source. The lasers do not then need to have a fixed position with regard to the microscope.
- A scanning head that contains the optics and mirrors to control the scanning.
- Various types of image recording or photon detection equipment (for example, photomultipliers or cameras).
- Computer control systems and viewing screens.
- Sometimes alternative light sources such as arc lamps.

FIGURE 3
Overview of a confocal laser scanning microscope



Source: Zeiss.

18. Confocal systems can often be upgraded to be able to use the multiphoton approach. The upgrade process involves fitting the infrared pulsed lasers and making changes to the software and lenses; in some cases substantial physical changes must be made to the systems to make this possible. Some confocal systems are described as 'multiphoton ready' or in similar terms. In general this means that it would be relatively easy to convert the system to use the multiphoton approach. Zeiss use the term to refer to those of its systems that can be upgraded on site, but other manufacturers and commentators may use such terms with different or less specific meanings.

19. Significant development has occurred in the methods of marking biological specimens with fluorescent dyes, which has been paralleled by developments in the microscopes. Confocal systems now often have more than one laser so that different features of the specimen can be marked in different ways and then separately identified. There have also been developments in detection systems that allow subtle distinctions between fluorescent markers to be identified.

20. A variety of advanced types of experiment have been developed, exploiting the capabilities of advanced systems. These include:

- FRET (fluorescence resonance energy transfer);
- FRAP (fluorescence recovery after photobleaching); and
- FLIM (fluorescence lifetime imaging).

These techniques allow scientists to study phenomena such as the interactions of proteins within living cells and the dynamics of protein movement. Additional equipment and software is typically required compared with a basic confocal system.

Product innovations in advanced 3D light microscopy

21. The first commercial confocal systems were brought to market in 1984 by Bio-Rad and Zeiss. At that stage, the emphasis was on material science applications. In 1987, Bio-Rad introduced the MRC 500 system, developed in collaboration with the UK Medical Research Council specifically for biological applications. In these early systems, the available computing equipment was much less powerful and sophisticated than it was to become, limiting the scope for data storage and processing.

22. Leica entered the confocal market in 1988, Nikon in 1993 and Olympus in 1996. Other suppliers also entered the market from 1988 onwards but did not always make a big impact. Many of these other entrants were exploiting a particular technological variant or market niche such as the various spinning disc systems. Over time the market has become deeper with products being developed with a wide variety of price/performance characteristics. Systems have progressively improved in terms of reliability, ease of use, flexibility and software function. Nevertheless, not all product launches have been successful, with a number failing for technical or marketing reasons. Bio-Rad, Zeiss and Leica have tended to be at the leading edge of the technical development.

23. Zeiss has drawn our attention to important technical developments over the years which include the following:

    (a) Leica introduced an Acousto Optical Tunable Filter (AOTF) in 1992 which gave much faster control of the laser source and thus reduced specimen damage.

    (b) In 1996 Bio-Rad introduced the MRC-1024 MP multiphoton system.

    (c) In 1998 Zeiss introduced the LSM 510 NLO multiphoton system.

    (d) In 2000 Leica introduced the TCS SP2 SL spectral system which had the capability to distinguish fluorescent markers with similar colours, giving biologists much greater scope in the markers they could use. This was a particularly important advance.

    (e) In 2000 PerkinElmer introduced the UltraView spinning disc system. This was marketed much more effectively than previous systems of this type and established a significant market presence.

    (f) In 2001 Zeiss introduced the LSM510 Meta, a spectral system using different technology to that of Leica and providing sophisticated software.

    (g) In 2002 Zeiss introduced their ApoTome system, using the structured illumination approach.

(h) In 2002 Bio-Rad introduced the Radiance Rainbow which has a spectral capability similar to that offered by Zeiss and Leica.

(i) In 2002 Leica introduced the TCS SP2 AOBS, responding to the Zeiss META system. This also incorporated an Acousto Optical Beam Splitter (AOBS); this allows more flexible use of lasers and use of different fluorescent markers.

(j) Leica also introduced a 'resonance scanning' system in 2002 for fast data acquisition.

(k) In 2003 Bio-Rad introduced the Cell-Map, a low-cost system with fewer facilities, aiming to meet a demand for low-cost confocal systems.

24. Table 1 shows the main confocal products as identified by Zeiss and goes beyond the above list.

TABLE 1 **Multiphoton and confocal laser scanning microscopes—models**

	Zeiss	*Bio-Rad*	*Diverse*	*Leica*	*Nikon (TIC)*	*Olympus*
1982	LSM Prototype					
1983						
1984	LSM 44	SOM 100				
1985	LSM 40, 41,42					
1986		MRC-500				
1987						
1988	LSM 10, 20	MRC-600	Phoibos 1000	CPM		
1989				CLSM		
1990			Sara. 2000, Odyssey			
1991	LSM 310, 321		Meridian InSight			
1992	LSM 410	DVC-250, MRC-1000	MultiProbe 2001	TCS 4D		
1993			Odyssey XL	TCS 4D AOTF	RCM 8000	
1994	LSM 410 AOTF	MRC-1024	MultiProbe 2010			
1995					TIC/PCM 2000	
1996		MRC-1024 MP	Noran OZ	TCS NT		Fluoview
1997	LSM 510		CSU-10	TCS SP		
1998	LSM 510 NLO	Radiance plus	Atto CARV	TCS MP, TCS E		
1999	LSM 5 PASCAL	μRadiance, 2000/MP				FV-500, FV-300
2000		RTS 2000, MP	UltraView	TCS SP2, SL		
2001	LSM 510 META	Radiance 2100, MP		ICM 1000	C1	
2002	ApoTome	Radiance Rainbow	CSU-21	TCS SP2 AOBS, RS		
2003		CellMap	Visitech VTi			DSU

Source: Zeiss.

Note: This table also includes spinning disc systems and the Zeiss ApoTome system, which uses structured illumination.