

Laser light illuminates medical research

Kylie M Price* and Mike Berridge

Malaghan Institute of Medical Research

PO Box 7060, Wellington, 6242

Introduction

Light in the form of lasers has played a key role in biomedical research and advancing human health over the past 61 years. Today, most people are familiar with lasers, but many do not fully appreciate the descriptive acronym: **L**ight **A**mplification by **S**timulated **E**mission of **R**adiation. The process called 'Stimulated Emission' that underpins lasers was first conceived and developed by Albert Einstein in 1917. Before delving into the use of lasers in medical research, a brief introduction to this revolutionary light source is warranted.

Lasers

Charles Townes and Arthur Schawlow invented the world's first laser-like device in 1954. However, as it emitted microwave radiation using ammonia gas as the gain medium it was called a 'maser'. It wasn't until 1958 that they published papers on the theory of using this technique to generate visible light, inspiring Theodore Maiman to build the first laser in 1960 [1].

Lasers have three special properties that separate them from other light sources: they emit beams of light that are coherent, monochromatic and have very little beam divergence. Light can be viewed as waves, radiating outwards from a source, in which each wave causes a fluctuation in the (usually null) value of the electric field at points through which the waves pass; coherence means that all of the waves are traveling together *in phase* – i.e. they are all in step (Figure 1).

The monochromatic nature of light from a laser means that it consists of a single wavelength and is therefore a spectral colour (i.e. one seen in a rainbow). The colour depends on the

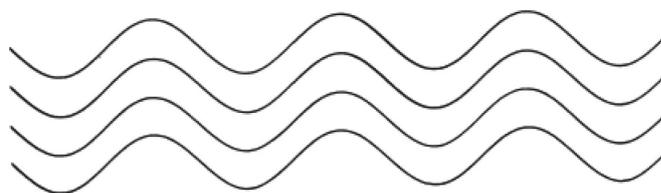


Figure 1. Coherent laser light, all wavelengths travelling in phase.

gain medium used to generate the light. Many different materials will support laser action and these can be in any state: gas, liquid or solid. For example a 530 nm laser pointer would be green (Figure 2).

One of the earliest red lasers (632.8 nm) used for its gain medium a 10:1 mixture at low pressure of helium and neon gases. The gain medium is excited by an external energy source, in the form of a high-voltage electrical discharge through the gas, which results in energetic electrons colliding with ground-state helium and neon atoms and exciting them to higher energy states. This process leads to a 'population inversion', i.e. more atoms with electrons in higher energy levels and vacancies in lower energy levels than vice-versa. In this situation, photons whose energy is equal to the energy difference of these levels can stimulate excited neon atoms to de-excite, emitting photons of the same wavelength (colour), propagating in the same direction and in phase (coherence) with the stimulating photon. Mirrors, one of them slightly transparent, at each end of a tube of the gases, reflect photons back and forth in the gain medium, stimulating repeated emission by the excited atoms and generating a light beam emitted through the partially transmitting mirror.

As a consequence of the repeated reflection between two parallel mirrors and the emission of photons in the same di-

*Correspondence: kprice@malaghan.org.nz

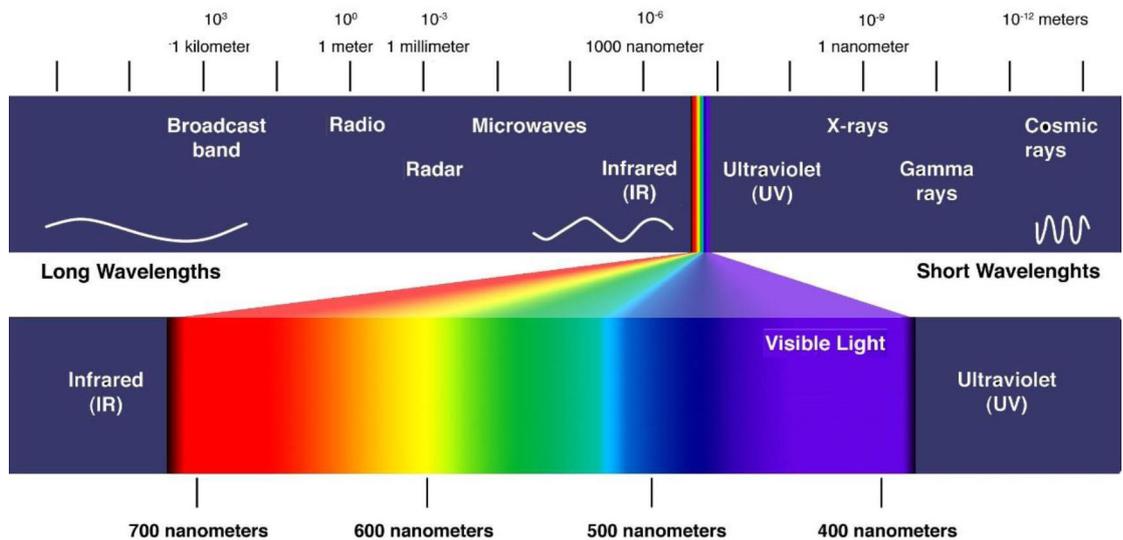


Kylie Price heads the Hugh Green Cytometry Core at the Malaghan Institute. She obtained a BSc majoring in Biochemistry from the University of Otago in 2000 and Master of Science in Cell and Molecular Bioscience from Victoria University in Wellington, New Zealand, in 2003.

Mike Berridge is a Distinguished Research Professor and Group Leader at the Malaghan Institute. He was a Postdoc at Purdue University and a Staff Scientist at the National Institute of Medical Research, Mill Hill, London, before returning to Wellington as the second Malaghan Research Fellow in 1976.



Figure 2.
The electromagnetic spectrum [2].



rection as the stimulating photon, lasers have very little beam divergence. This means that laser light persists as a single beam of light over long distances, compared to a standard hand torch, for example, where the beam divergence is much greater.

These three properties make it possible to focus the well-collimated and intense beam of laser light through a lens, and it is this feature in particular that makes lasers so powerful for medical research applications and scientific discovery. Being able to illuminate live or fixed cells or even parts of cells or particles allows us to explore the properties and functions of cells, their membranes, and the organelles that reside within them.

Flow cytometry

At the Malaghan Institute the two main technology platforms that use the power of laser light are Flow Cytometry and Confocal Microscopy. The starting point for the modern flow cytometer was the 'Coulter Counter' (patent filed by Wallace Coulter in 1949), which enabled automated counting and sizing, initially of red blood cells (RBCs), based on electrical impedance. In 1953, Crosland-Taylor discovered a method to inject a suspension of particles into a faster moving stream of fluid flowing in the same direction, thus putting the 'flow' into flow cytometry [3].

In 1965 Mack Fulwyler was working in Los Alamos attempting to monitor fallout from atmospheric nuclear weapons testing to see if particles turned up in meal, milk and other food products. He was also trying to find out whether a population of RBCs that showed a distinctive bi-modal distribution using the Coulter Counter, was in fact two separate cell populations. Fulwyler read a paper by Richard Sweet, who had developed a device that produced a jet of ink in air (the concept behind ink-jet printing) [4]. The jet was vibrated so that the inkjet broke into droplets, and when these droplets were electrically charged, they could be deflected into collecting tubes. Fulwyler understood that injecting cells into a liquid stream that was vibrated to break into droplets, coupled with Coulter volume sensing to measure particle size, could provide a way of physically separating cells of interest [5]. By the mid-1960s the first flow cytometer capable of sorting cells had been built, but it was not until the 1970s that these instruments became commercially available.

Flow cytometry also utilises lasers to stimulate fluorescent dyes that are attached to cells of interest, usually via an antibody specific for a cell marker. Cells are injected into a liquid stream

and hydrodynamically focussed such that each cell passes in single file (Figure 3) through the laser beam, whose width is usually 1–2 cell diameters.

As cells traverse the laser beam, their physical characteristics such as size and surface texture are measured by light scatter, and fluorescent signals from each cell are determined. The photons that are emitted from a fluorescent compound after laser excitation are detected by an array of highly sensitive photomultiplier tubes (PMTs). PMTs act as the 'eyes' of the flow cytometer, their role being to take a single photon of light that gets through filters, amplify the signal in a linear fashion that will depend on the voltage applied, and convert the light detected into an electrical signal that is proportional to the number of photons incident on the PMT. In this way, fluorescent probes bound to specific molecules on and inside cells are measured as they pass through the laser beam.

A successful flow cytometry experiment requires cells of interest to be extracted from tissues or blood as a single cell suspension so that individual cells can be interrogated by the lasers. The power of flow cytometry lies in the speed with which cells can be analysed and the sheer volume of information that can be collected on each cell. For example, cells can be run

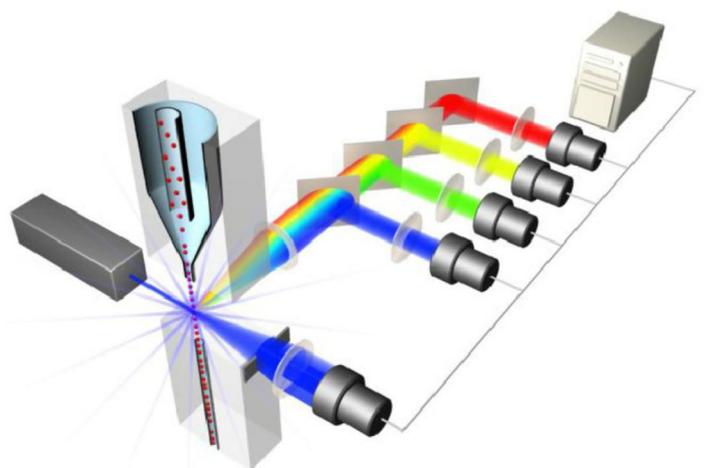


Figure 3. Schematic of a flow cytometer [6]. Cells pass through a laser beam in single file. The varying emission wavelengths of the fluorescent dyes attached to the cell are sent to PMTs via an array of mirrors and filters allowing each PMT to detect a single colour. These signals are sent to a computer, where they are processed and digitised for analysis.

through a flow cytometer at 20,000 cells per second, at which speed physical scatter properties and up to eighteen distinct fluorescent parameters can be collected for individual cells and different cell types isolated by Fluorescence-Activated Cell Sorting (FACS).

In flow cytometry, laser light is used to stimulate the fluorescent proteins and organic molecules (collectively called fluorophores) to re-emit light. These fluorophores, often attached to antibodies, are highly specific in their binding kinetics, e.g. for the CD4 T-cell receptor. When a cell with a fluorescently-labelled antibody attached passes the laser, the electrons of the fluorophore move into an unstable, short-lived, excited state. To return to the stable ground state the excited molecule gives off a photon of light, producing the 'signature colour' of that fluorophore. Another characteristic of fluorescence is that the light emitted is nearly always of a longer wavelength and lower energy than that of the light which excited the fluorophore. This is due to small energy losses which occur while electrons are in the excited state (Figure 4).

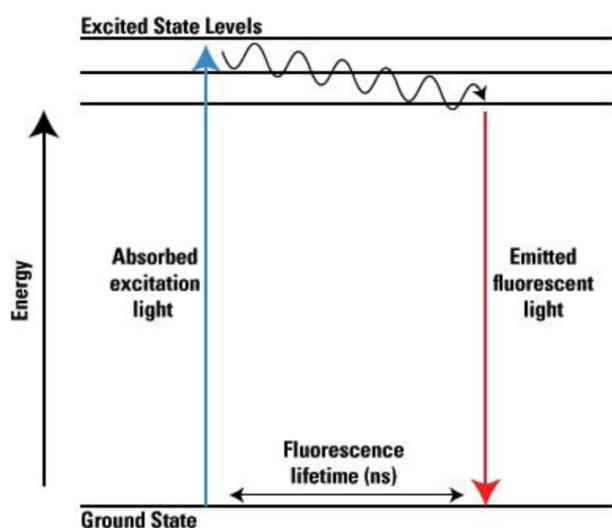


Figure 4. Jablonski energy diagram of fluorescence [7].

Fluorophores are generally grouped into three categories: proteins and peptides, small organic compounds, and synthetic oligomers and polymers. An example of a naturally occurring fluorescent protein commonly used in flow cytometry is Green Fluorescent Protein (GFP). This protein was originally extracted from the jellyfish *Aequorea victoria* found in the Puget Sounds in Washington State in 1962. GFP is optimally excited by a 488 nm (blue) laser and has an emission maximum around 509 nm meaning it emits green light. GFP or another fluorescent protein can be attached to a receptor or cytokine (chemical messenger) within a cell or animal by genetic manipulation. In cells where the receptor or cytokine is being expressed, its presence can be measured and its location determined by fluorescence microscopy.

Using flow cytometry, complex immunophenotyping experiments are possible that include determining the functional status of specific cells of the immune system. For example, it is possible to determine whether immune cells have encountered foreign material (antigen) in the past and whether they are memory or functional effector cells, whether they are quiescent or senescent, dividing or dying, and it is even possible to determine where a cell of interest originated from, for example, the skin.

Cell sorting

The first cell sorter or FACS machine in New Zealand, a BD FACS 420, was installed at the Christchurch Clinical School of Medicine in about 1980 while the Wellington Cancer and Medical Research Institute (renamed the Malaghan Institute of Medical Research in 1986) and Wellington Hospital purchased a similar instrument in 1983 facilitated by a substantial personal donation from Mrs Ann Malaghan [8]. This machine had two large high-powered, water-cooled lasers and was installed in a special purpose, air-conditioned hospital laboratory adjacent to the Research Institute. We 'borrowed' software from Christchurch to run our FACS 420. Visits to Len and Leonore Herzenberg's laboratory at Stanford University, where leading-edge developments in cell sorting were in progress, and training at the Becton Dickinson production facility in Palo Alto ensured rapid uptake and timely clinical application of the developing technology, initially for leukaemic blood cell analysis.

The Malaghan Institute has the most advanced multilaser flow cytometers and cell sorter in New Zealand with the cell sorter (BD Influx) and one cytometer having six lasers: ultraviolet, (355 nm), violet (405 nm), blue-violet (445 nm), blue (488 nm), green (532 nm), and red (640 nm) and another cytometer having five. For these lasers, more than 100 dyes and tandem dyes are now available and the list is growing rapidly. The larger number of lasers per instrument means that more sophisticated 'questions' can be asked about cells in the sample. If the sample happens to be a one-off patient sample such as blood, tissue or tumour biopsy, then more information can be obtained on the immune cell status relative to disease progression, and this information used to help direct treatment.

Cell sorting utilises a principle involving the electrostatic deflection of charged droplets similar to that used in ink-jet printers [9]. Cells injected into a liquid stream in air break into droplets, but droplet formation is unstable. To overcome this issue, cell sorters use a stationary wave of vibration produced by a piezo-electric crystal, to produce a stable break-off position to generate droplets. Cells are injected into the liquid stream from a nozzle which can be changed to control the size of the stream and therefore accommodate sorting cells of different sizes. Cells in droplets pass the laser beam for interrogation. If a cell meets the sort criteria (i.e. has the markers/receptors of interest), it will be identified for sorting [9].

The time taken for a cell to pass the laser and end up at the point at which the cell breaks off from the solid stream into a droplet is calculated and known as the drop delay. With this knowledge, the cell sorter can be instructed to charge a droplet when it breaks off from the stream. These charged droplets then pass between two high-voltage deflection plates. If a droplet is positively charged it gets drawn towards the negatively charged plate and into a collection vessel and *vice versa*. If a droplet is uncharged it will be aspirated to the waste (Figure 5). By controlling the concentration of cells being injected into the stream it is possible to ensure one cell for every four droplets formed.

Cell cycle sensing

There are also highly sophisticated systems and fluorescent probes that can report specific cellular activities, which can be detected by both flow cytometry and fluorescence microscopy. One example is a cell cycle sensor system called Fluorescence Ubiquitination of Cell Cycle Indicator or FUCCI. This is a

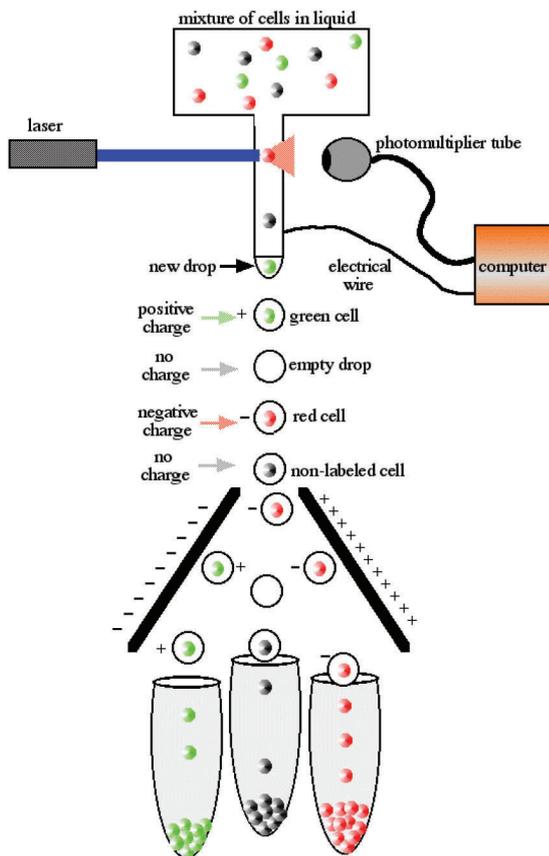


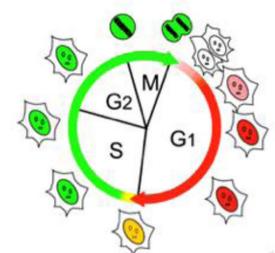
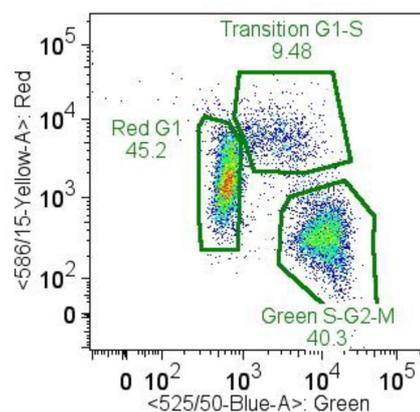
Figure 5. Schematic of a jet-in-air cell sorter or FACS machine [10]. Droplets containing cells of interest break from the core stream. Charge is attached depending on the cell phenotype (e.g. positive charge to droplets containing green-labelled cells and negative charge to droplets containing red-labelled cells). Droplets with no charge are separated to waste (black-labelled cells).

plasmid-based probe that allows real-time analysis of transitions between phases of the cell cycle [11].

The cell cycle is a highly regulated series of events that ultimately results in one cell dividing into two identical daughter cells. The cell cycle is also where the all-important DNA repair occurs. If a cell sustains DNA damage, for example due to free radical attack, cell division is arrested to facilitate excision and repair. Many human cancers involve mutations or over-expression of key proteins that control cell cycle progression, allowing cancer cells to overpower these checkpoints and divide in an uncontrolled manner that is associated with accumulating DNA damage. Being able to monitor, understand and control cell cycle progression is of vital importance to cancer researchers.

A system like FUCCI allows light to be used to track cell cycle progression. Two anti-phase oscillating proteins tagged with different fluorescent proteins are used to monitor different phases of the cell cycle: cells will appear red in G1 (repair and preparation for DNA synthesis), green in S, G2 and M (DNA synthesis and prior to cell division) and colourless immediately following M (Figure 6). Another key feature of this system is that as cells transition from G1 to S phase they appear

Figure 6. Left panel: Flow cytometry dot plot showing the three distinctive populations of cells (G1, Transitioning G1-S and S/G2/M). Right panel: Schematic of the fluorescent protein expression pattern of FUCCI labelled cells [11].



yellow. All of these states can be seen by both fluorescence microscopy and flow cytometry, and if necessary, cells can be isolated in any of these phases by Fluorescence Activated Cell Sorting. Pure populations of cells transitioning through the cell cycle can then be further investigated to determine exactly which genes are being turned on or off at each step.

Confocal microscopy

For many years, light has been used to elucidate cellular behaviour and pathways of disease. The earliest developments were in the world of optical microscopy where a light beam is used in conjunction with lenses to provide a magnified image of a sample. There are many different types of microscopes ranging from compound instruments where the objective lens is close to the sample, to inverted microscopes for imaging cells in culture flasks. Stereomicroscopes generate 3D images of larger specimens like insects and worms. The confocal microscope is a specialised microscope that enables researchers to look deep within fresh or fixed cells or tissues of interest. It generates 3D images that give not only *context* but also the ability to peer inside cells to see their inner makeup. Powerful multi-photon instruments allow real time imaging within tissues like the skin, brain and lymph glands of live animals. Confocal microscopy uses a bright point of light, usually a laser, to illuminate the sample. Light is focussed onto a pinhole located in front of the detector (also a PMT as in flow cytometry), which rejects out-of-focus light from the sample (Figure 7). This increases the optical resolution and contrast, allowing for multi-dimensional observations of cell and tissue morphology (shape) and precise molecular localisation. The bright illumination spot, the fluorescent spot, and the pinhole are all in **conjugate focal planes**, hence the name 'confocal'.

An example of how confocal microscopy facilitates our research is the role it plays in understanding the dynamic interplay between immune responses and the microbiota in the gut. It allows visualisation of tight junctions between epithelial cells, which are the gatekeepers of gut tissue. These cells create a physical barrier between the contents of the gut and body tissues. Changes in this barrier function are being investigated as they initiate chronic inflammatory diseases of the gut.

The optical precision of the confocal microscope and multi-photon microscopy allows fluorescent markers to be used to identify particular types of immune cells within gut tissue. Cells involved in immune responses are quantified and their location and interactions with other immune and non-immune cells explored.

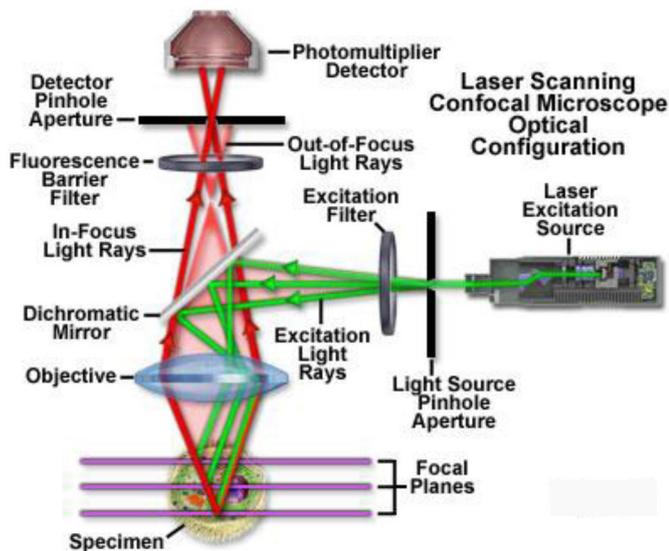


Figure 7. Schematic of the light path in a confocal microscope [12].

Recent developments

Many factors have changed over the years to allow such profound and rapid advances in both flow cytometry and microscopy. Alongside the technological developments discussed, there were the important discoveries of antibodies in the 1970s, which led to the production of humanised monoclonal antibodies in the late 1980s and now to the use of highly specific tetramers and dendramers capable of tagging the cell of interest with exquisite specificity. There have also been significant developments in fluorescent technologies from the earliest fluorophores extracted from natural sources through to the development of light-harvesting fluorescent polymers that led to the 2014 Nobel Prize in Physics [13].

The much-discussed laser has also undergone significant changes over the past fifty years; lasers are now smaller (Figure 8), cheaper, more powerful and available in just about any colour of choice. In addition, the constant evolution in digital capabilities and the speed and processing power of computers



Figure 8. During the upgrade to the new BD Influx cell sorter in 2014, Kylie Price compares the old red laser on the table to a new red laser in her hand. The laser being held is 8x more powerful (120mW compared to 15mW) than the previous laser and 20x smaller. First generation water-cooled lasers were several times the size of the larger laser pictured.

facilitate faster passage of cells through flow cytometers with more information being collected about each cell. With microscopy, high-resolution images are digitized, and multiple images of the same section taken at different depths can be stitched together (Z-stacked), displayed in 3D and rotated.

Light underpins flow cytometry and microscopy and as these technologies advance and develop, so too does our understanding of cells and the immune system that protects us. The use of light is allowing the Malaghan Institute to push the boundaries of knowledge and understanding in our fight against cancer, asthma, allergies, autoimmune diseases like multiple sclerosis, neurological diseases and parasitic diseases.

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Additional reading

- <https://www.youtube.com/watch?v=o2joszUiVhM> Introduction to flow cytometry webinar.
- Shapiro, H.M. 2003. *Practical Flow Cytometry*, 4th edn. Wiley.