# HOW CELLS DIE

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Faculty of Health Sciences

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INAUGURAL ADRESS

Of

Professor Lissinda du Plessis

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## Abstract

Flow cytometry is a technology that measures and analyzes the optical properties of mono-dispersed single particles, such as cells, bacteria, picoplankton, microbeads, yeast, platelets, nuclei and other similarly-sized particles, passing single file through a focused laser beam. The laser can excite fluorophores that have been used to mark various molecules or physiological functions of the particles. The use of fluorophores with different fluorescence characteristics, multiple lasers and multiple photo detectors allows flow cytometers to measure many characteristics of each particle simultaneously. An important feature of flow cytometry is that large numbers, for example thousands of particles per second, are analyzed and therefore provide a statistically significant picture of a specimen's physical and biochemical make-up. In this inaugural address the focus will be on how we implemented flow cytometry in cellular toxicology or the study of how cell dies.

## Introduction

The historical origin of the word “inaugural” is said to be derived from Latin augur or augurare, which refers to rituals that Roman priests performed to determine if it was the will of the gods for a public official to take office. In the early 1980’s the title of the inaugural address read in Afrikaans as “Rede uitgespreek by die aanvaarding van die amp as Hoogleraar….”. It is therefore with these historical guidelines that I have the honour of giving my inaugural address tonight. I will begin by giving some background on my development as a researcher and then move onto a historical perspective on how cells are analysed. I will then present a selection of my research findings and summarise at the end.

## My roots in research

I sometimes see myself as the proverbial “Jack of all trades, and master of none”, which in this case would rather be master of some. My undergraduate training was in Physiology, Zoology and Biochemistry. I continued with Honours and Master’s degree in Physiology. Circumstances at the time lead me to a PhD in Biochemistry. During this time my promoter, Prof Harry Kotzé gave me the opportunity to learn flow cytometry. I still recall the midnight drives to Orkney for analysis of samples. At that stage the NWU had purchased the flow cytometer, but it had yet to be delivered, but the research had to continue. This in turn gave me the opportunity to start a post-doctoral fellowship at Pharmaceutics under the mentorship of Prof Awie Kotzé, where the flow cytometer was housed. I could additionally hone my skills in flow cytometry to become the experienced analyst I am today. I greatly appreciate the expert mentorship, opportunities and guidance I received from the professors Kotzé.

## Flow cytometry at the North-West University

In 2006, the NWU had purchased the FACSCalibur from BD Biosciences, their Rolls Royce at the time. In 2011, BD announced that they would be discontinuing the FACSCalibur and we received strategic funding to purchase its replacement the FACSVerse. In 2012, Physiology received strategic funding for the FACSAccuri a portable flow cytometer enabling researchers

to take flow cytometry on the road. Towards the end of last year we decided to centralise all the flow cytometers in the Faculty of Health Sciences and they are housed just across from us in the laboratory (G20, LAMB).

**Jack of all trades?**

Although my training seems like major different fields there is however a common thread that I followed to get there that can be described by cellular toxicology. Toxicology is defined as "the study of the harmful actions of chemicals on biological tissue". It is further stated that it involves an understanding of chemical reactions and interactions by understanding biological mechanisms (Klaasen, 2001). By understanding these mechanisms at cellular level we can extrapolate the findings to the entire organism. It is with that in mind that I will present to you my passion in research, **How cells die**. This field is also known as Necrobiology.

**The basics**

I would first like to explain some of the basics. The **cell** is the fundamental unit of life and is defined as a discrete collection of chemical entities that has the ability to self-replicate. It is important to remember that cells do not exist in isolation but are organized into specialized tissues to make up an organism or human (Klaasen, 2001). When cells die the most notable changes occur within the nucleus, mitochondria or the plasma membrane. By studying cells, we can infer important information on diseases and how to treat them.

**Cytometry**

The study of cells can include many strategies but one important aspect involves measuring the properties of cells. This field of study is called **cytometry**. Cytometry is a process in which physical and/or chemical characteristics of single **cells (cyto-)** in roughly the same size range are **measured (-metry)**. The properties of cells that we can study includes what type of cell it is, functional characteristics (how a cell works) and structural characteristics (how a cell looks). Historically scientists studied cells by looking at them through a microscope, but with time newer technologies were developed. Different types of cytometers were developed

including image cytometers, cell sorters, time-lapse cytometers and flow cytometers. **Flow cytometry** is the measurement of properties of cells in a flowing system (Shapiro, 2003). Flow cytometry is a newer cytometry technology, although it is no longer "new". However, to obtain a clear understanding of flow cytometry as a technology, we have to briefly go back in time.

**Historical perspective**

The starting point is light and the electromagnetic spectrum (Fig 1). It is a range of frequencies (the spectrum) of electromagnetic radiation and their wavelengths. For our interest we only look at a small portion of the spectrum, the light spectrum where we have visible light and ultraviolet (UV) light. We use light sources, either visible or UV, to illuminate cells in order to study their properties (cytometry).

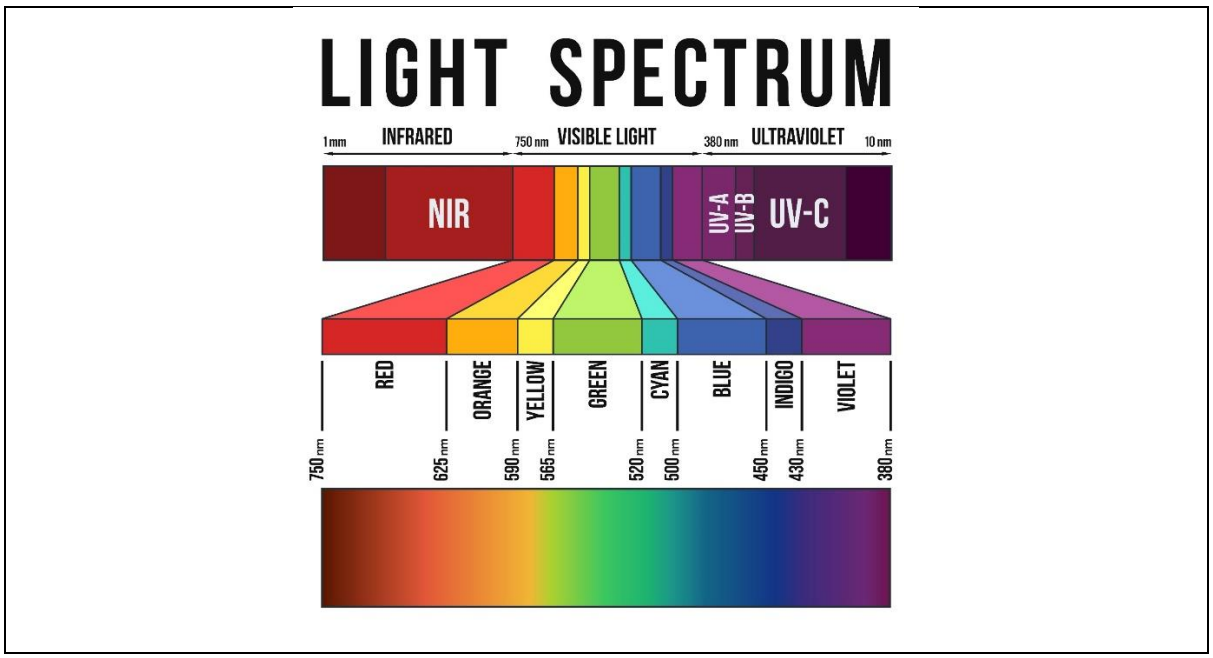



**Figure 1** A portion of the electromagnetic spectrum illustrating the light spectrum that consists of different types of light at different wavelengths in nanometres (nm).

**Phase 1 Visualising cells**

The first curiosity among scientists was simply just being able to see cells (Fig 2). Image cytometry is the oldest form of cytometry. The first measurement of cells that was published,

dates back to the description of the microscope being used to visualise red blood cells by Antonie von Leeuwenhoek in 1674. This enabled scientists to see cells in limited detail by optical microscopy using **visible light**. Although cells could be observed, there was a need for improved resolution to observe cells in greater detail. In 1904, German scientists (August Köhler, Moritz von Rohr at Carl Zeiss) developed the first **ultraviole**t (UV) microscope. In essence they replaced the visible light source with a UV light source. The shorter wavelength improved the resolution of the cells being observed, but they reported another unknown light reflection (fluorescence) as a nuisance after UV illumination. Advances in theory in the early twentieth century enabled the development of fluorescent microscopes. Otto Heimstädt, working at the optical company Carl Reichert (later became part of Leica), and Heinrich Lehmann, working at Carl Zeiss developed the first fluorescence microscopes independently as an outgrowth of the UV microscope (1911-1913) (Zannachi *et al*., 2014). All of these discoveries enabled scientists to **visualise** the properties of cells in great detail. A modern day representation of how cells look under light microscopy, illuminated with UV light and fluorescently labelled cells are shown in Fig 2.

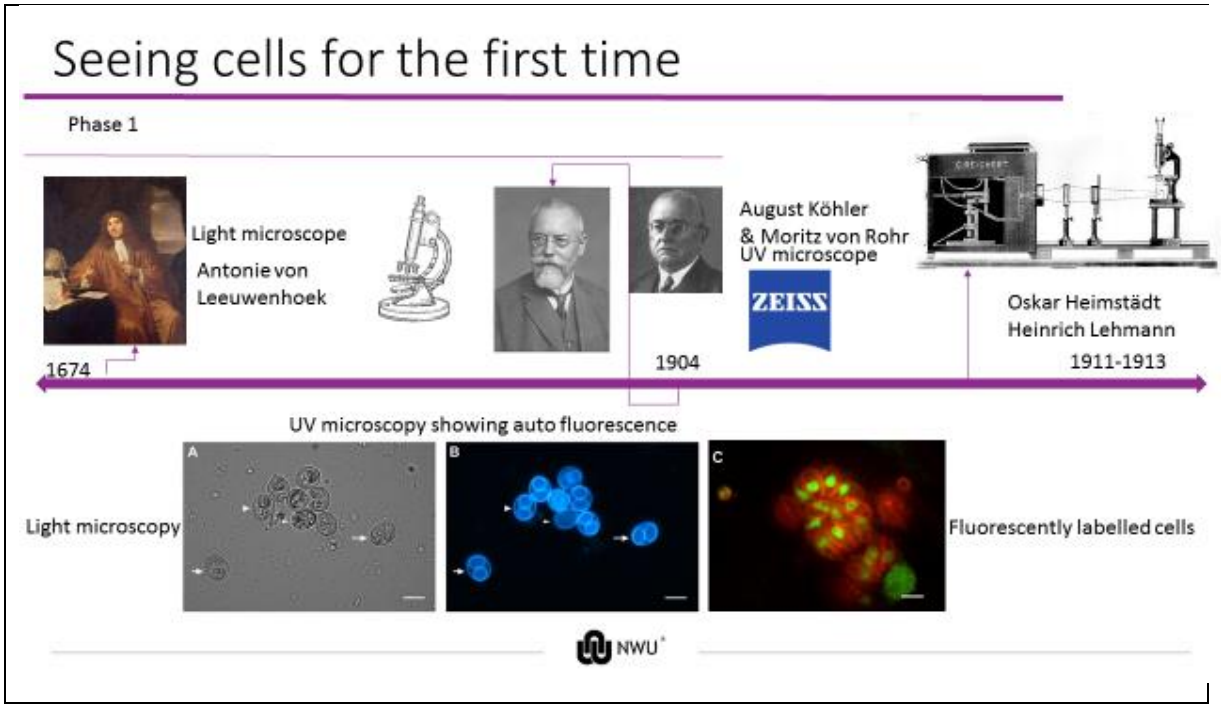



**Figure 2** The historical timeline of the development of microscopy. The bottom part show a modern day image of cells as viewed under a microscope illuminated with A) visible light and

B) UV light and C) stained with fluorescent dyes. (Microscope Image credit of https://toxophysics.wordpress.com/autofluorescence/).

Scientist however did not only note fluorescence during UV microscopy. In the 16th and 17th century scientists already started noting **fluorescence**, although that was not the name they used. The most notable advancement in fluorescence came from George Gabriel Stokes, an Irish scientist, in 1852, when he described the term dispersive reflection on the phenomenon of the antimalarial drug **quinine** sulphate, he coined the term fluorescence (the famous Stokes shift in fluorescence is named after his discovery) (Fig 3). Quinine was the standard antimalarial treatment and prophylaxis in the 17th and 18th century (Achan *et al*., 2011). Most of you might be familiar with the historical description of British officials stationed in India. The quinine powder was too bitter for them and to increase compliance the mixed the powder with soda water and sugar, the birth of Indian tonic water. It was also popular to mix it with gin where the popular gin and tonic drink developed. Adolph Von Baeyer (1871) a German chemist, synthesized a chemical compound (Spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one, 3',6'-dihydroxy), which he named "fluorescein", from "fluo" and "resorcin" (recorcinol). He won the Nobel Prize in chemistry in 1905 for his work on organic dyes. Paul Elrich, a German physician, made notable advances in staining cells, especially blood cells and received a Nobel Prize in physiology and medicine for his work on immunity (1908). He also did the pioneering work on staining cells with methylene blue and proposed it as an antimalarial drug at the time (Zanacchi *et al*., 2014). A photo of Paul Elrich in his office is included, where the stacks of papers and chemical can be seen (Fig 3).

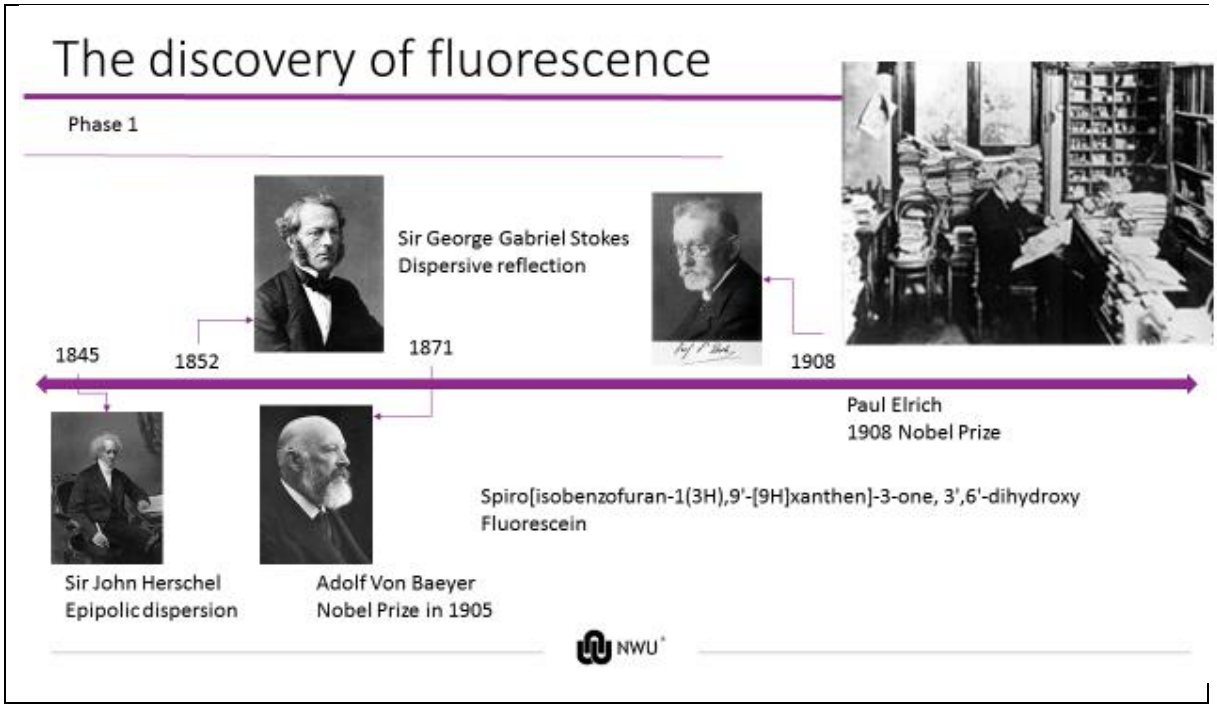



**Figure 3** A historical timeline that illustrates the most notable developments in the discovery of fluorescence.

**Phase 2 Counting cells**

During the late 19th century various scientists also started noting the importance of not only seeing cells, but also counting cells, especially blood cells. The counting of cells could be used to diagnose patients and optimize their treatment. Various scientists developed the haemocytometer, which is a blood counting chamber, together with a light microscope used for counting cells, manually. However, the task is laborious and marked by human error and the need developed for automatic cell counting. By the 1930's, many companies were manufacturing fluorescent microscopes. A modern marvel, the spectrophotometer, was developed in 1940 by Arnold Beckman at his laboratory (Beckman instruments) which would become the company Beckman Coulter we know today (Robinson, 2013). A spectrophotometer used a light source, various mirrors and a quarts prism and a phototube detector. The visible light is split into different wavelengths, that directed by mirrors fall on the sample. The manner in which the solution in the sample absorbs this light, gives it a unique absorbance spectrum. This absorbance spectra is used to identify the chemical substance. Shortly after this, scientists combined fluorescent microscopes with spectrophotometers and

named it cytophotometers (Fig 4). These instruments focused on counting the number of particles suspended in fluid from light scatter.

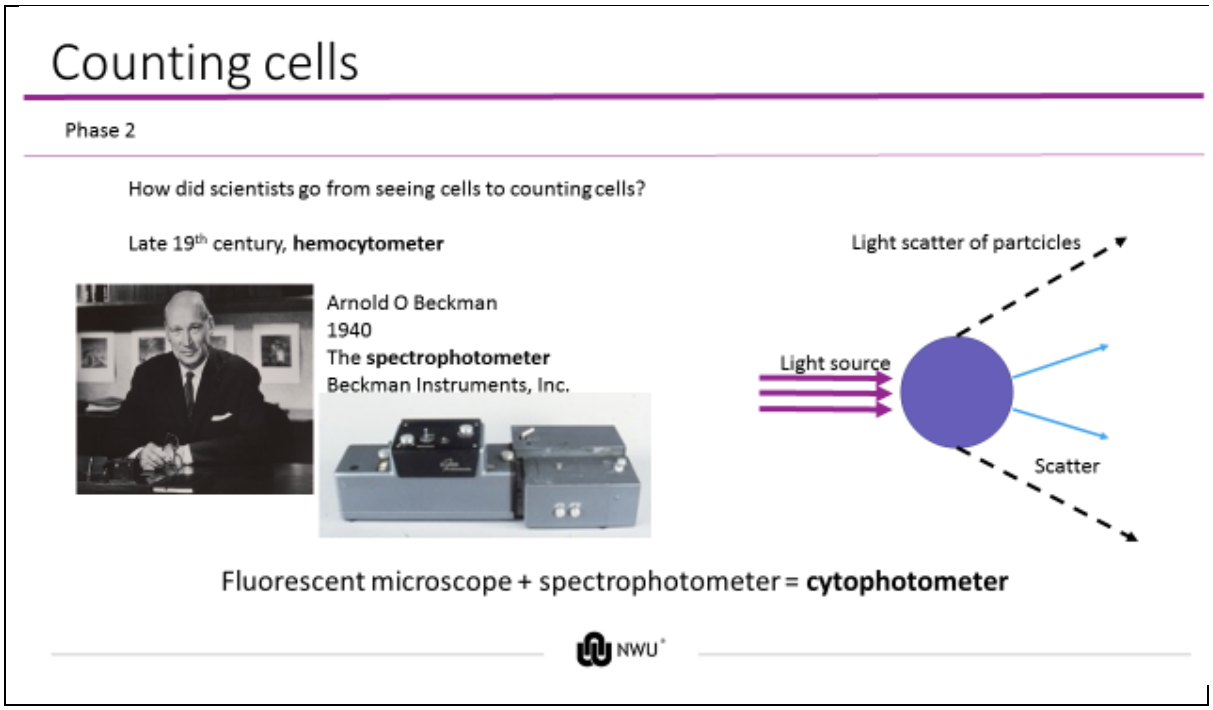
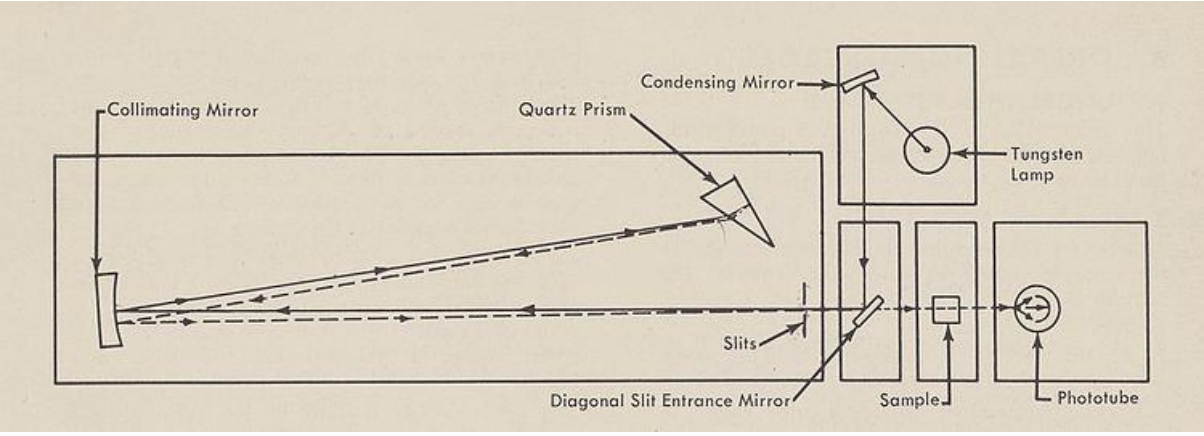



**Figure 4** The development of counting cells with different types of instruments in the late 9th and early 20th century.




**Phase 3 Development of flow systems**

Scientists increasingly worked on the automation of cell counting. The very first attempt was published in 1934 by Andrew Moldovan, which used a microscope, cells being forced through a capillary tube and a photoelectric device that created a micro-current each time a cell passed by (Moldovan, 1934). The difficulty remained in how to precisely align the cells to be counted accurately. In the late 1940's Wallace H Coulter developed the principle of using electrical impedance/conductance to count and size particles suspended in a fluid, passing

through a small orifice (The coulter principle). He established the Coulter company. In 1998 the company merged with Beckman instruments and it became the Beckman Coulter company (Fig 5). This was the first commercial flow system for measuring cells (Simson, 2013). (The coulter principle and the coulter counter still used today).

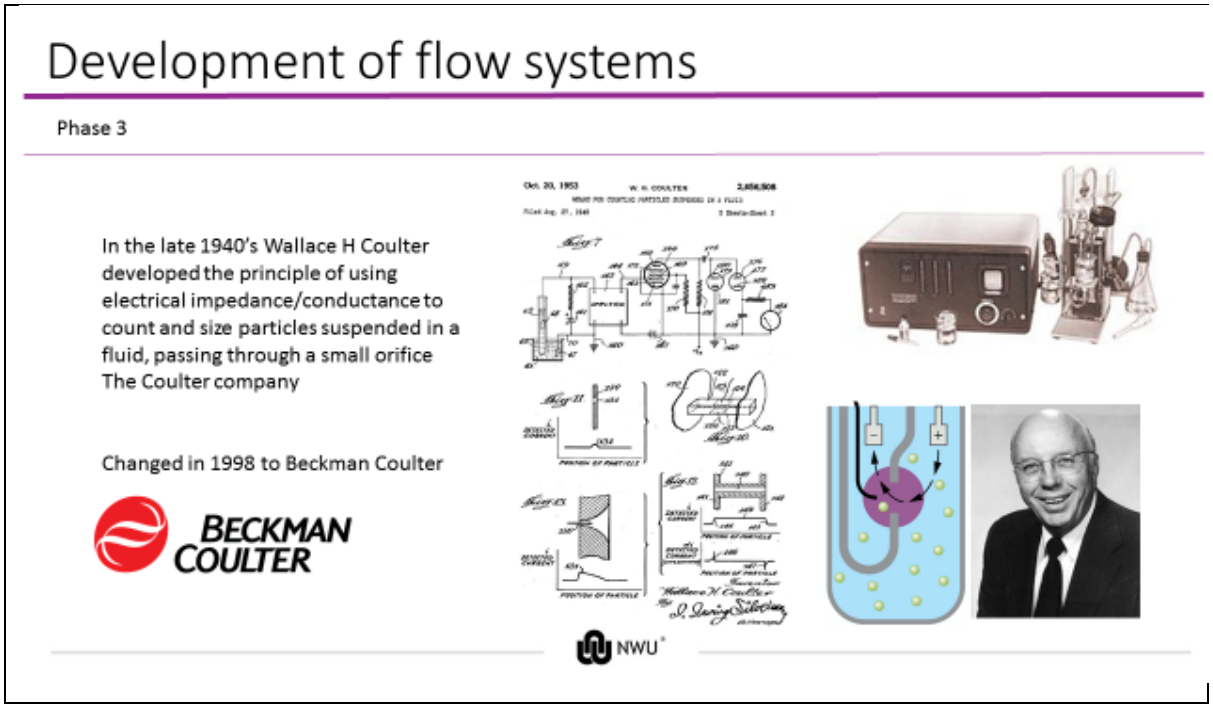



**Figure 5** The development of measuring cells in flowing systems, illustrating the original hand drawn patent of Wallace Coulter and the original coulter counter. An image is also included to illustrate the electrical conductance principle.

**Phase 4 The birth of Immunofluorescence**

At this time Albert Coons (1941) labelled antibodies with fluorescein isothiocyanate (FITC), thus giving birth to the field of immunofluorescence. Most of the current techniques still employ this principle in which the fluorescein derivative is coupled to a secondary antibody that binds to the primary antibody. The primary antibody would bind to the target on the outside or the inside of a cell (Fig 6). Cells could now be more accurately typed and classified into different morphologies, a major breakthrough in cancer diagnostics (Coons *et al.*, 1941).

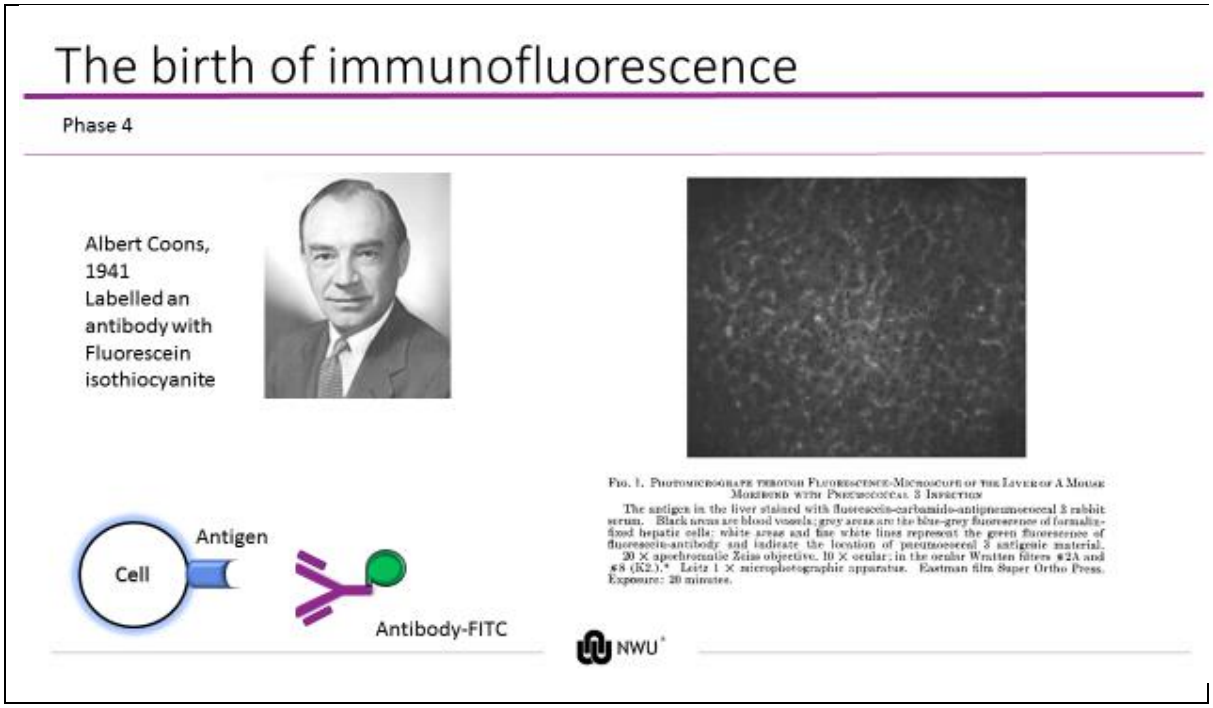



**Figure 6** The original figure published by Albert Coons, illustrating the staining of cells with fluorescein isothiocyanate (FITC).

**Step 5 Combining it all into a Flow Cytometer**

In 1953, P.J. Crossland-Taylor published an unsuccessful attempt to count red blood cells by aligning cells by using sheath fluid to hydrodynamically focus the cells, visualising cells with dark field microscopy (Fig 7). He described it as "slowly injecting a suspension of the particles into a faster stream of fluid flowing in the same direction. This principle did not use capillary tubes. The principle was proven, but it needed improvement (Crossland-Taylor, 1953). In the late 1950's and early 1960's computers was introduced to many laboratories and mathematical modelling were implemented in medical diagnosis. Statistical methods were computerised enabling scientists to work with larger sets of numbers accurately. Pattern recognition were implemented in cell identification where the optical density was pixelated. Louis Kamentsksy with experience in statistical and optical methods for cell recognition developed the first rapid cell spectrophotometer. This combined a microscope, with a light source, optics with a microscope slide and cells passing through on at a time, with computers. The cells could now be seen on a computer screen and image processing were possible, measurements based on a biological parameter could now be made automatically (Kamentsky *et al*., 1965). Mark Fulwyler combined the Coulter principle with continuous inkjet

technology (then newly developed) to create the first cell sorter in 1965. It included droplet formation, when a stream of fluid emerging from an aperture is hydro-dynamically unstable and breaks into a series of droplets – the same principle of physics used in inkjet printers. They were able to sort the different types of blood cells automatically (Shapiro, 2003; Picot *et al*., 2012). Therefore at this stage researchers were able to count and sort cells automatically, by using a combination of spectrophotometers, light microscopes, hydrodynamic flow and computers.

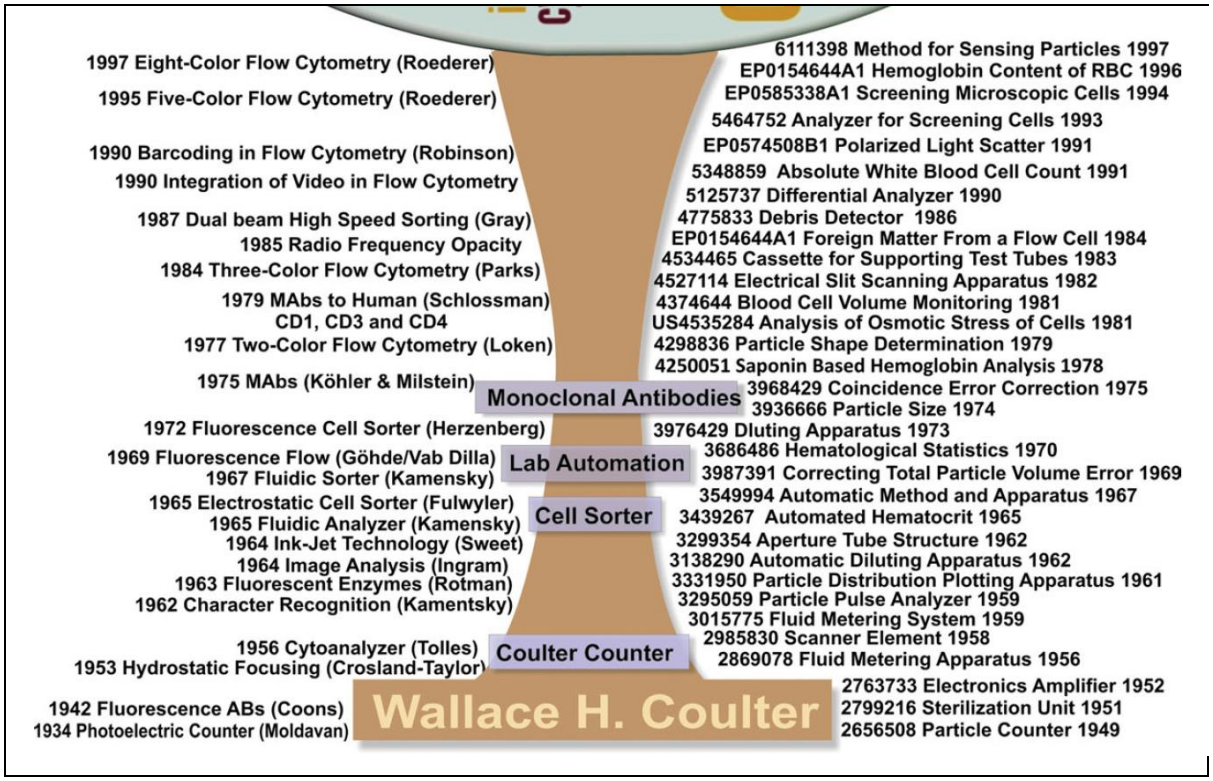



**Figure 7** Timeline figure with modern developments, including the patents issued to Wallace coulter on the right (Robinson, 2013).

**Step 6 The development of FACS**

The first fluorescence-based flow cytometry device was developed in 1968 by Dittrich and Göhde, which they named pulse cytophotometry. In the late 1970's scientist could measure DNA content of cells for the first time by using fluorescent probes with a flow cytometer and soon other parameters followed. Various flow cytometers were commercially developed and in 1974 the first fluorescence activated cell sorter (FACS as we know it today) were developed by Leonard Herzenberg at Becton Dickinson (BD Biosciences today). In 1972 the method of

cell death apoptosis was coined, which enabled cytometrists all over the world to combine flow cytometry with the interesting research field of how cells die. In 1978 the name pulse cytophotometry was officially changed to flow cytometry, which became the umbrella term including pulse cytometry and FACS. In the early 1970's it was still built around a fluorescence microscope and two parameters could now be measured, including visible light scatter and fluorescence (one colour). In the 1980's benchtop flow cytometers were introduced (before this a flow cytometer could almost fill a room) by BD biosciences, only the optics of the microscope remained. Two colour flow cytometry were first described in 1977, three colours were used in 1984 for the first time. Apoptosis were first studied with flow cytometry in the early 1990's and became the method of choice (Darzynkiewicz, 2018). The focus then moved to not only using flow cytometry in diagnostics, but also in basic research. More advancements were made and five colour flow cytometry were introduced in 1995 and 8 colours only in 1997 (Shapiro, 2003; Robinson, 2013). Scientists could now see cells, count cells, sort cells and characterise cells by using 10 parameters measured at the same time. To better illustrate the principle of multi-parameter analysis, we have to briefly review the basic principle of flow cytometry.

**The basic principle of flow cytometry**

Step 1 Light and illuminating cells – the optics.

A beam of light, usually from a laser, is directed onto a hydrodynamically-focused stream of fluid containing cells or particles. Light travels in waves, if we go back to light spectrum and wavelengths. As light travels it might encounter other waves of objects that allows the light to scatter. The next step is to be able to see the light, this is where we use optics, similar to the ones found in microscopes. Here we divert the light from where it wants to go to where we want it to go.

Step 2 Fluidics.

The fluidics transport the cells from the sample to the measuring station. Under a microscope the cells are stationary, but here we use hydrodynamic focussing. This is using a stream of

fluid (sheath fluid) to confine the sample fluid to a central portion. This improves the precision at which a cell can be positioned for analyses. Pressure or vacuum pumps drives the fluid, as is the case in a squeeze bottle.

Step 3 Alignment of light.

This is where we collect the light that was emitted or scattered from the cell in the fluid and directing it with various filters and mirrors to where it can be detected.

Step 4 Detecting light.

The next step is the detectors that are aligned at different angles to the point that the fluid stream passes through the light beam. One in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter (SSC) and one or more fluorescent detectors). The detectors that we use included photodiodes and photomultiplier tubes. Photodiodes can be compared to solar panels that produces a current when exposed to light. Photomultiplier tubes also works like this, but the current is multiplied by using a power source. This is used for weaker signals such as fluorescence. The detectors detect light even if a cell is not passing by and this is the background current. When a cell passes by, the light scattering changes and a current pulse is observed. The characteristic of the pulse provides information on the cell (Adan *et al*., 2016).

Step 5 Electronics.

Each suspended cell passing through the beam scatters the light in some way or fluorescent markers attached to the particle may be excited into emitting light. These combination of scattered and fluorescent light is picked up by the detectors. The current generated by the detectors are converted to analogue signal and then to digital signal. We use acquisition software to process the signals and we measure different properties/parameters based on dotplots and histograms (Shapiro, 2003). It is important to note that all the properties of each individual cell is measured at the same time enabling us to (based on the amount of lasers and detectors) measure up to 20 cell properties at one time. The flow cytometer can also count up to a million cells in a matter of minutes, providing information on each individual cell. In contrast with a spectrophotometer one response is measured of the entire population of cells (Shapiro, 2003; Picot *et al*., 2012). We can measure the size of cells (FSC), the

complexity of cells (SSC) and almost anything that a fluorescent probe or fluorescently labelled antibody in a cell can bind to. The FACSVerse at the NWU has a 2 laser, 6 colour configuration or 6 detectors and can measure 8 parameters simultaneously (size, complexity and 6 fluorescent parameters). The most recent flow cytometer launched in 2016, are able to be customised with 10 lasers and 50 detectors, allowing for the measurement of 20 parameters simultaneously.

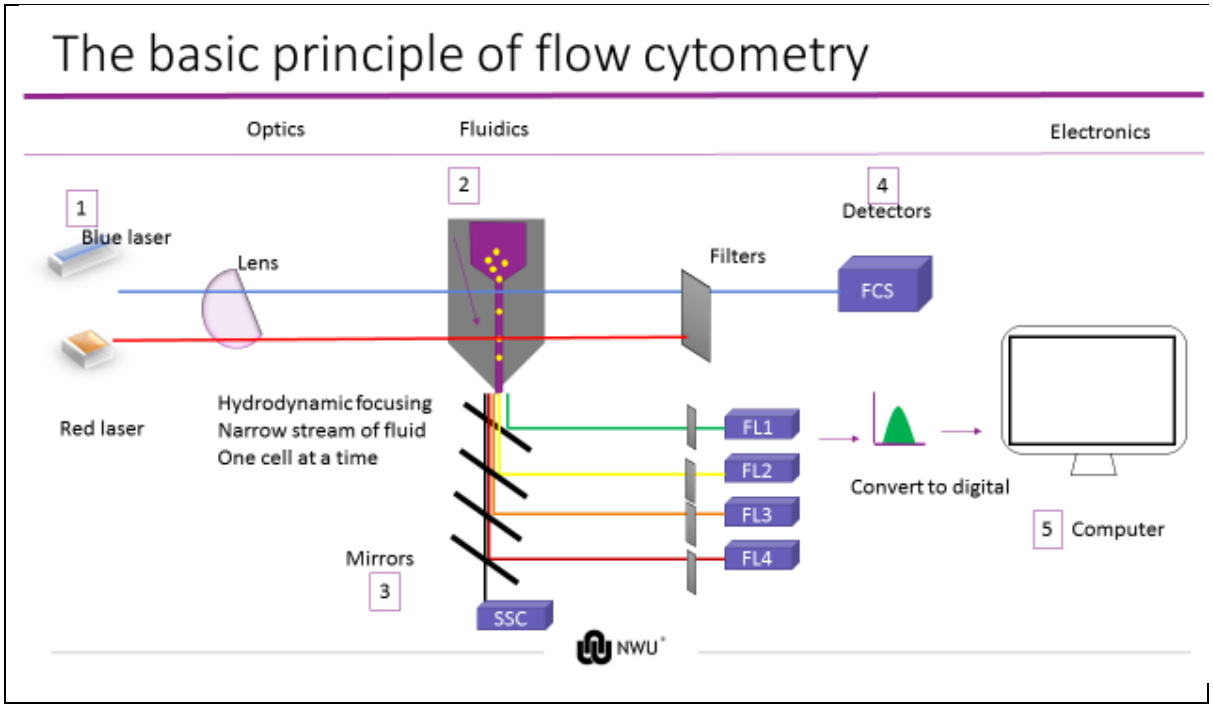



**Figure 8** A diagrammatical presentation of the different components of a flow cytometer.

By measuring the properties of cells we can detect whether a cell is alive, dying or dead. We can also elucidate the mechanism of how cells function normally, during chemical insult, in pathological states or in response to drug treatment. This brings us to How cells die.

**How cells die - necrobiology**

Well believe it or not but scientists are still studying this and proposing different mechanisms (with the glory behind every new mechanism being proposed), with 34 types of cell death described up to date (Liu *et a*l., 2018).

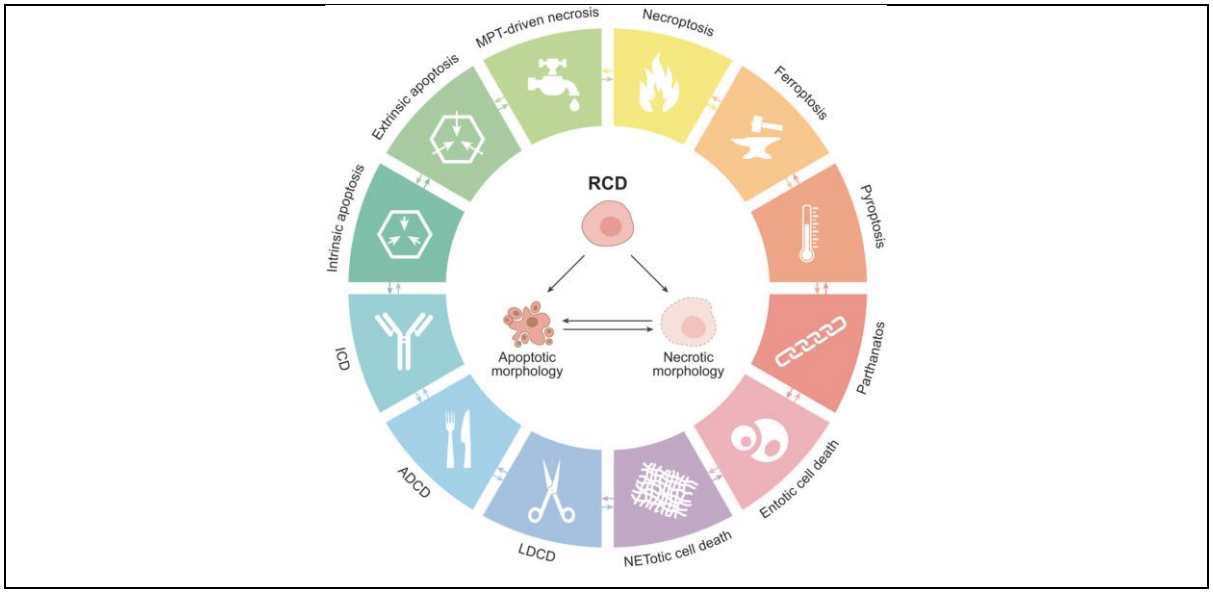



**Figure 9** The different types of regulated cell death classified into 12 sub categories and group under two main categories.

However, there are actually a Nomenclature Committee on Cell Death that has formulated guidelines in 2018 for the definition and interpretation of cell death from morphological, biochemical and functional levels. These are grouped into two categories regulated cell death and accidental cell death (Fig 9). Regulated cell death is seen as a physiological process that occurs as a targeted elimination of irreversibly damaged or potentially harmful cells. Accidental cell death is the spontaneous and catastrophic demise of cells exposed to severe insults of physical, chemical or mechanical influences (Galluzzi *et al*., 2018). We can compare this to Dr Jekkyl and Mr Hyde; apoptosis being Dr Jekkyl, the calculated scientist carefully planning his approach like a sniper and controlling it in great detail; and necrosis being Mr Hyde that enters the seen triggering an unplanned bomb explosion. In apoptosis there are certain biochemical events that are triggered and a highly regulated cascade of events leads to the cell death where the cell shrinks (Fig 10). Clinically when there is excessive apoptosis we observe atrophy and when there is insufficient apoptosis it leads to cancer. In necrosis it is uncontrolled and messy, where the cell swells the plasma membrane rupture and the cell content leaks usually leading to inflammation. Clinical examples of necrosis are usually due to external stressors, like extreme cold or a spider bite. To further illustrate my Dr Jekkyl and Mr Hyde theory of how cells die, I would like to highlight a few of the studies that I worked on. I have to state that none of these studies would have been possible without the opportunities provided by co-workers and hard work of students under my guidance.

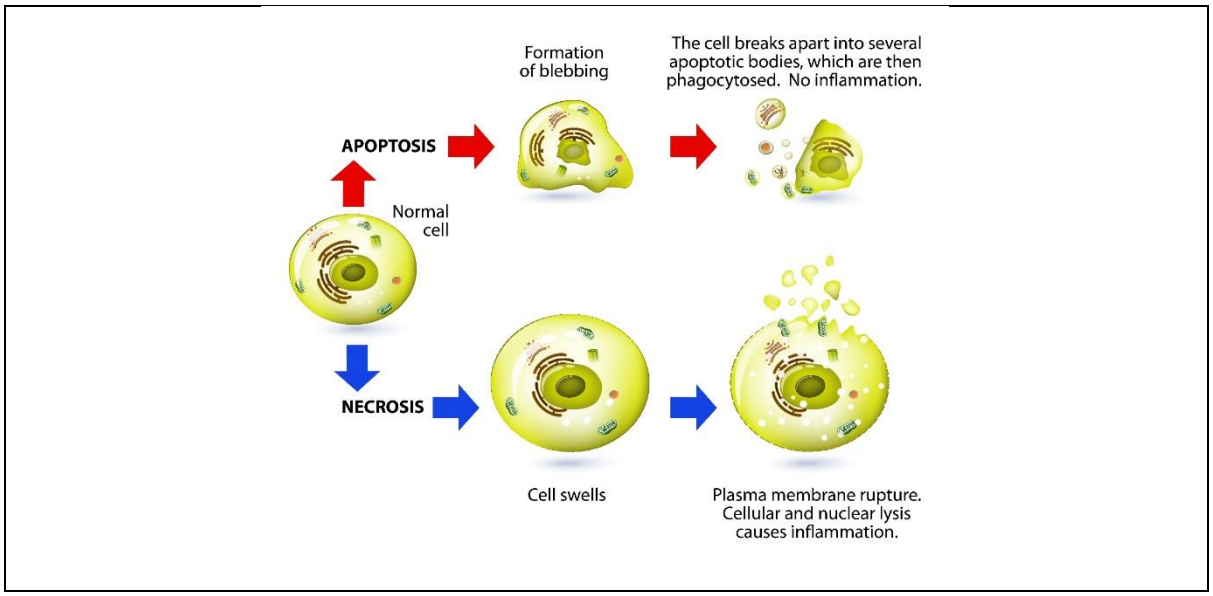



**Figure 10** The main physiological and biochemical changes that occur when cells die.

**Properties of cells that we study**

The first property of cells that we measure with flow cytometry is the cell size. Information of cell size is supplied by the forward scatter of the cell. We mostly make use of dotplots, where each dot on a graph is representative of a cell (Fig 11). We can see this on a dotplot, the smaller the cells the lower they will be on the scale and larger cells will be higher in the scale. The next parameter is the complexity or granularity of the cell. Simple cells with very few organelles or particles in them will be on the lower scale and more complex cells will be on the higher scale. This is nicely illustrated by the dotplot of whole blood where simple small red blood cells containing only a nucleus lies at the lower left side of the plot and large nuclei neutrophils lies in the bottom right corner (Du Plessis *et al*., 2010). The complex granulocytes can be found in the upper right corner.

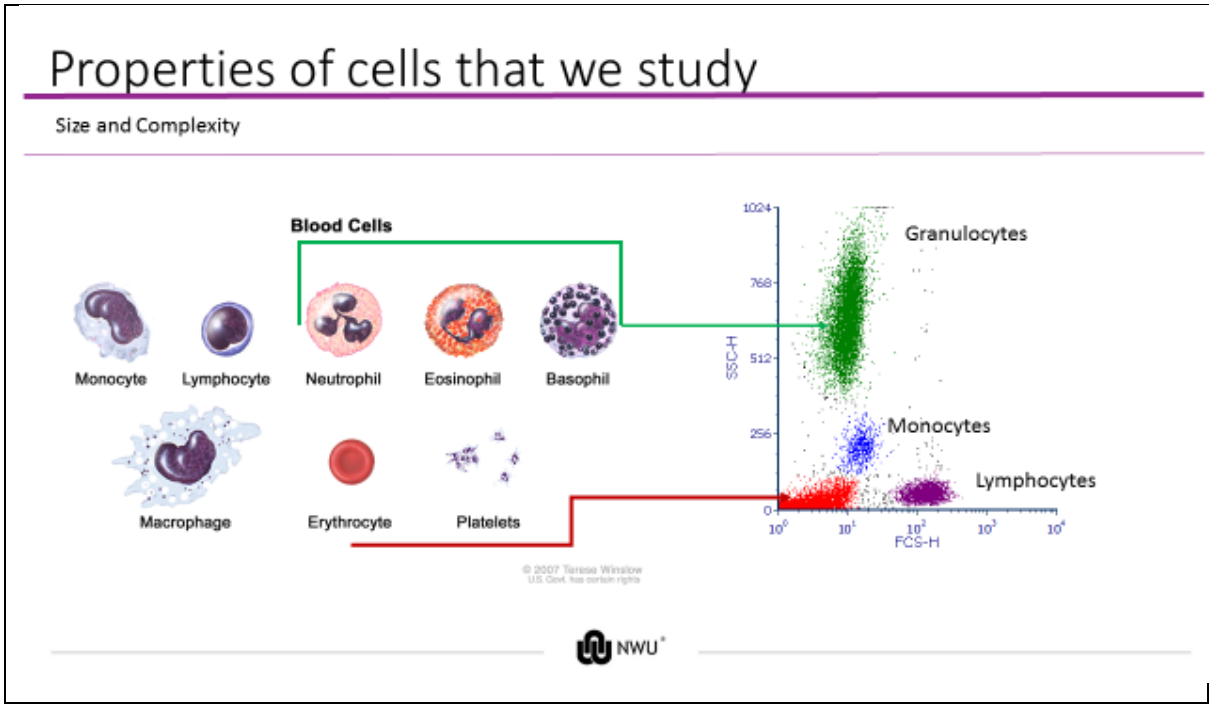



**Figure 11** The different morphologies of the different types of white blood cell and how it relates to the scatter pattern observed in the dotplot.

The next parameter we measure is fluorescence or where we use the jargon colour. We have seen that the modern flow cytometers are able to detect up to eight colours simultaneously. Fluorescence is a function of light energy. If we go back to quinine. When light passes through the quinine molecule light is absorbed by one colour (wavelength) and emits the light at another colour. The difference in these two peaks is the Stokes shift, named after Stokes that described it for the first time. In simpler terms we can say that a molecule absorbs blue light and emits green. It is similar for fluorescein or FITC, it absorbs blue light and emit green (Fig 12). We also use R-phycoerythrin (PE) which is a photosynthetic pigment found in red algae, conjugated to antibodies. It is excited at 490 nm and emits red fluorescence at 580 nm. The two axis of the dotplot is representative of the fluorescence intensity. The more of the dye bind to a cell, the more intense (bright) the fluorescence will be. In fluorescence analysis we divide the dotplot into four quadrants. In two colour flow cytometry cells negative for both dyes will be located in the bottom left corner, cells positive for colour A can be found bottom right, cells positive for colour B is top left and cells positive for both A and B will be in the upper right corner. This is perhaps best explained by selected examples of results from my research.

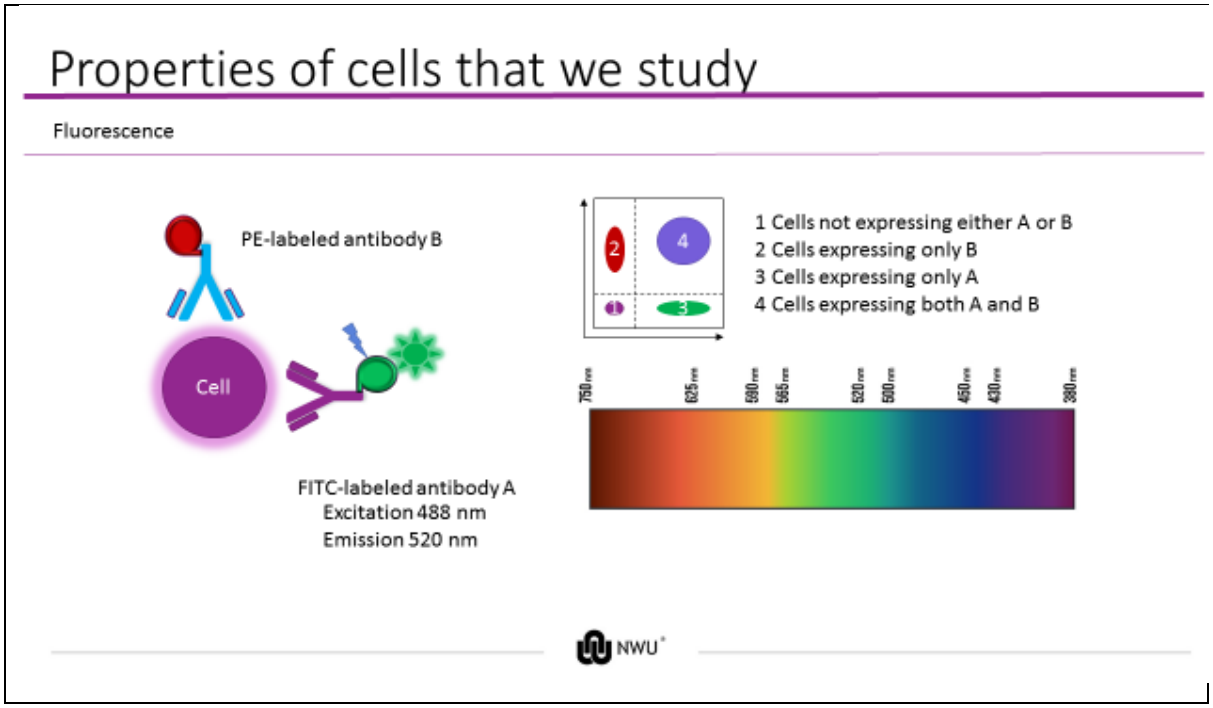



**Figure 12** A schematic presentation on how fluorescence is analysed by flow cytometry and how the data is interpreted.

We were able to distinguish between malaria infected red blood cells (upper right) and uninfected red blood cells (lower left) based on size and fluorescence (Fig 13). One can clearly see the difference in scatter between the un-infected red blood cells that do not contain any nuclei and the malaria infected red blood cell (Slabbert *et al*., 2011; Du Plessis *et a*l., 2012; Du Plessis *et al*., 2013). Additionally we included various DNA dyes for additional detection (Suzuki *et al*., 1997). The red blood cells dot not contain nuclear DNA and do not stain positive for the dye. The DNA dyes are cell permeable and are taken up by the infected red blood cells, staining the parasite DNA resulting in fluorescence. The larger the parasite, the more DNA it has and the higher the fluorescence intensity.

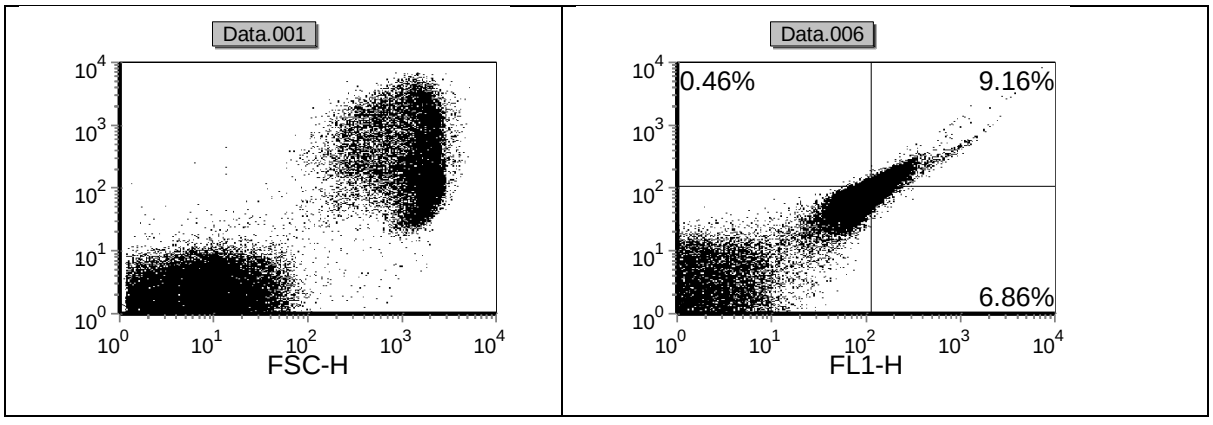


**Figure 13** The example of analysing *Plasmodium falciparum* infected red blood cells to determine the percentage of infected red blood cells.

We also used size and complexity to measure cell death (Fig 14). In necrosis the cells swell and in apoptosis the cells shrink (FSC) (Wlodkowic *et al*., 2011; Andon & Fadeel, 2012). We did various studies where we investigated the role of oxidative stress on DNA damage and different modes of cell death. In these studies we use hydrogen peroxide as a control to compare harmful substances to. In the untreated sample we see the scatter of 143 B cells (bone osteosarcoma), a model for mitochondrial deficiencies (Levanets *et al*., 2011). When treated with hydrogen peroxide, apoptosis is induced and the cells shrink and loses intracellular organelles or complexity. Additionally we use an antibody for Annexin-V and an impermeable DNA dye to further distinguish between different phases of apoptosis (Rieger *et al*., 2011). Annexin-V bounds to phosphatidylserine, an apoptotic marker that translocate from the inner to the outer plasma membrane during early apoptosis. This is represented on the dotplot by the cells in the lower right quadrant. During late apoptosis the cell membrane gets damaged, leading to a necrotic morphology and impermeable DNA dye, propidium iodide (PI) is able to enter the cell and bind to the DNA. This is represented by the upper populations. With this model we were able to characterise various metabolic and biochemical effects of metabolites.

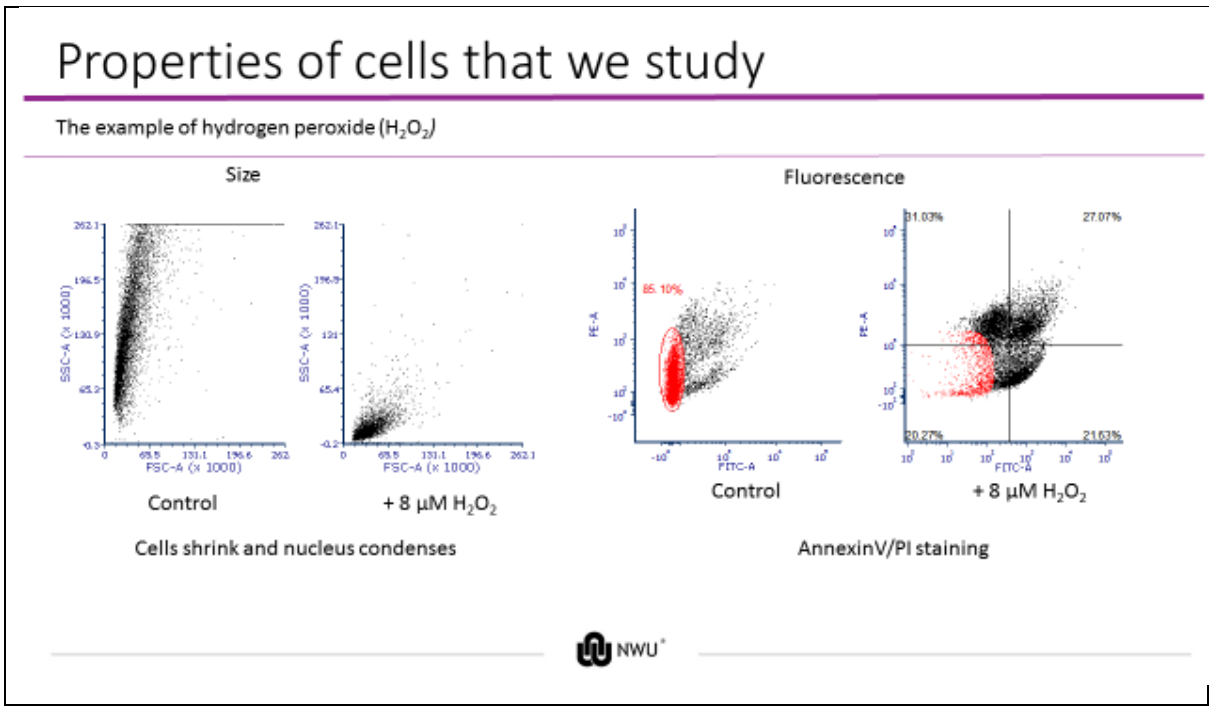


**Figure 14** Representative dotplots that illustrate how we use size, complexity and fluorescence to characterise various stages of apoptosis.

**Accidental cell death – Toxicology dose response**

No chemical is entirely safe and likewise no chemical agent should be considered as entirely harmful. This concept is based on the premise that any chemical can be permitted to come in contact with a biological mechanism without producing and effect on that mechanism provided the concentration of the chemical is below minimal effect level. The single most important factor that determines the potential harmfulness of safeness of a compound is the relationship between the concentration of the chemical and the effect that is produced upon the biological mechanism. No effect versus death, there must be a graded effect somewhere between the two extremes, called the dose-response relationship. Toxicants kills cells at low concentrations, whereas non-toxic compounds exert almost no effect on cells at high concentrations (Klaasen, 2001). I was involved in many studies observing the toxic effects of various compounds.

To illustrate this I would like to highlight a few of the studies that we did with excipients investigated for their potential to enhance oral drug absorption (Fig 15). Trimethyl chitosan chloride (TMC) is a carbohydrate polymer and a proven drug absorption enhancer. Mellitin an antimicrobial peptide from bee venom, also investigated as a potential drug absorption enhancer. These substances are classified as drug absorption enhancers because they are able to transiently open tight junctions (the spaces between cells) of intestinal cells allowing drugs to be absorbed between cells, rather than across cells. They are however toxic at high concentrations or prolonged exposures leading to extensive plasma membrane damage and necrotic cell death (Jacobs, 2015). With these studies we are able to suggest safety margin were these excipients can be successfully used in dosage forms, where toxic effects will not be observed (Du Plessis & Hamman, 2013).

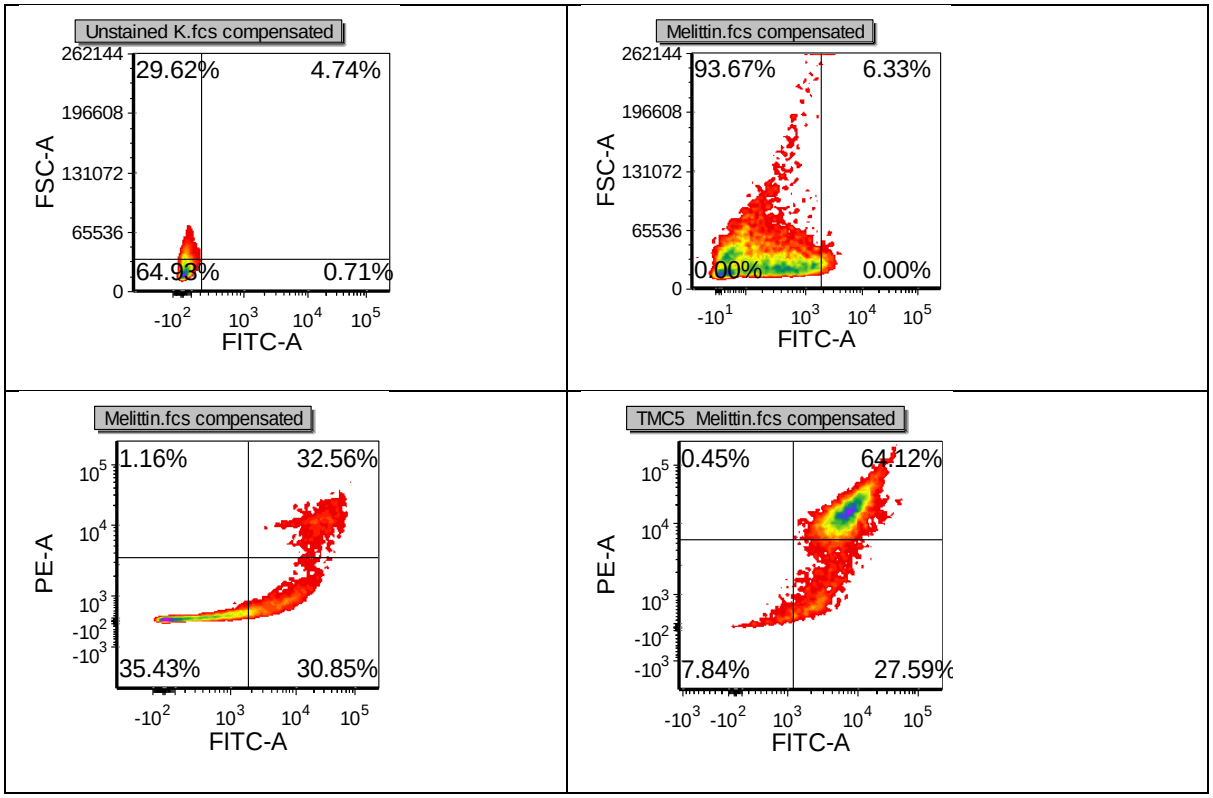



**Figure 15** Representative dotplots of necrosis in the presence of absorption enhancing excipients trimethyl chitosan (TMC) and melittin.

We can also use necrotic cell death to our advantage in killing micro-organisms (Fig 16). In these studies we were able to measure the influence of increasing antimalarial drug concentrations on parasites. The decreasing parasite levels can be observed with increasing drug concentrations. We did various studies with different anti-malarial drugs in various different formulations. All these studies lead to our current research where we are developing oral dosage forms for malaria treatment. I also collaborated with chemical engineering where we looked at using nanoparticles in killing bacteria in waste water (De Klerk *et al*., 2017). In these figures you can observe the increase in bacteria with significant membrane damage that were able to take up the DNA dye, propidium iodide (Stiefel *et al*., 2015). *Salmonella typhi* is the bacterium responsible for causing typhoid fever and removing it from waste water is essential for human health.

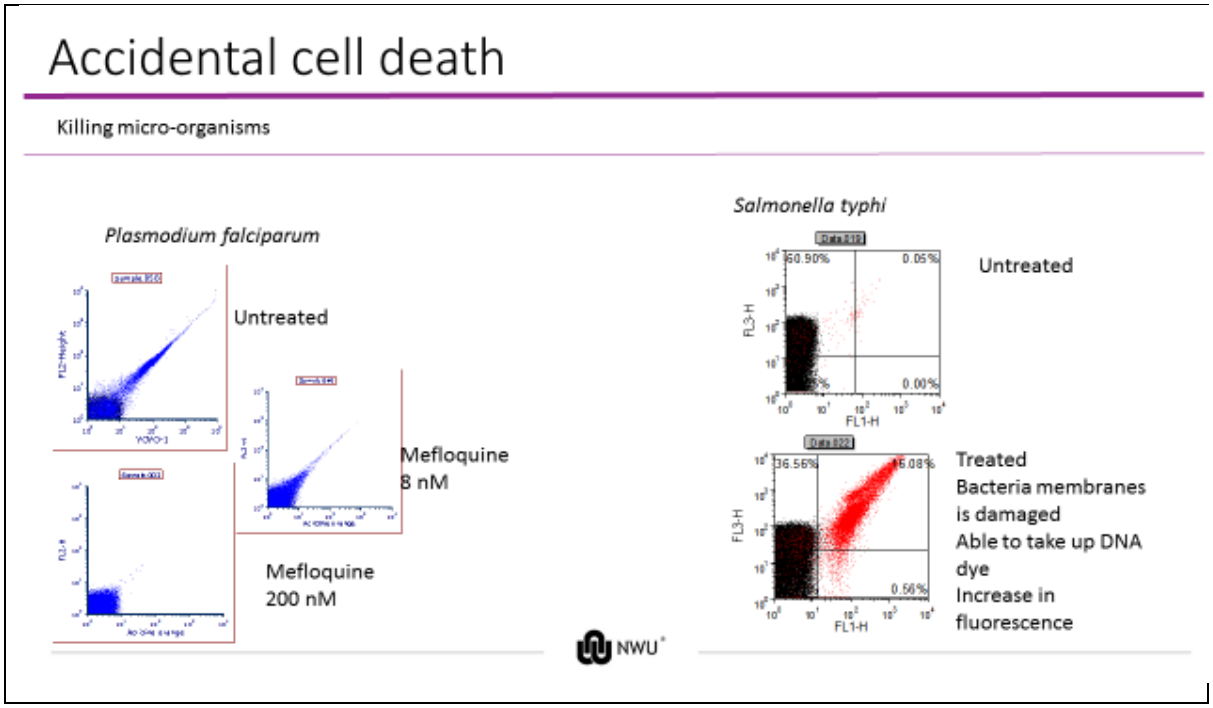



**Figure 16** Using necrosis to our advantage in killing micro-organisms.

**Regulated cell death**

In collaboration with nutrition we investigated the influence of mycotoxins on cellular toxicity (Fig 17). In this study we systematically investigated various parameters in regulated cell death including changes in the plasma membrane, nucleus and mitochondria (Wentzel *et al*., 2017a). These toxic secondary metabolites are produced by a range of fungi and are common contaminants of agricultural crops. Human can ingest large quantities of these mycotoxins when maize or rice are there staple diet. The mycotoxins induced apoptotic cell death, causing aberrant inflammatory signalling that will most likely be a contributing factor that initiates cancer in humans. An example of the membrane damage in apoptosis are shown in Fig 17. Worldwide effort are made to decrease the amount of these mycotoxins in the crops. We also did studies on various other substances that humans are exposed to either externally or due to normal metabolism that induced apoptotic cell death (Du Plessis *et al*., 2010; Bronkhorst *et al*., 2016a; Bronkhorst *et al*., 2016b; Wentzel *et al*., 2017b).

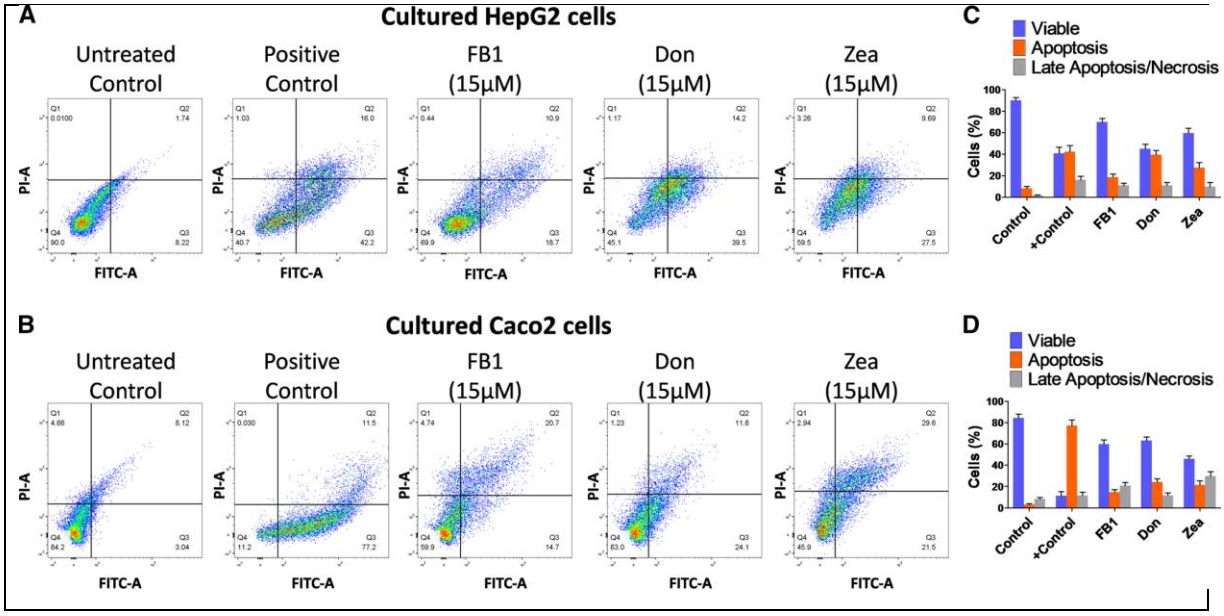



**Figure 17** Culture HepG2 and Caco 2 cells exposed to varying concentrations of mycotoxins. The different stages of apoptotic cell death, based on cell membrane permeability and translocation of phosphatidylserine is illustrated in the graphs.

Drug delivery vehicles can also influence the topical delivery and the efficacy of chemotherapy drugs. Together with Prof Richard Haynes we investigated the applicability of artemisone a novel anti-malarial drug in the treatment of melanoma (Dwivedi *et al*., 2015). We examined the encapsulation of artemisone in nanovesicular niosomes and solid lipid nanoparticles, and have evaluated efficacies of the free and encapsulated artemisone against human melanoma A-375 cells and effects on human keratinocytes (HaCaT). The formulations displayed highly selective cytotoxicity towards the melanoma cells with negligible toxicity towards the normal skin cells (Fig 18). The artemisone-loaded nano-vesicles almost completely inhibited the melanoma cells compared to the free drug. We have investigated various novel compounds and drug delivery systems in the treatment of cancer, in which regulated cell death plays a crucial role (Joubert *et al*., 2014; Chinembiri *et al*., 2015; Lewies *et al*., 2017; Lewies *et al*., 2018).

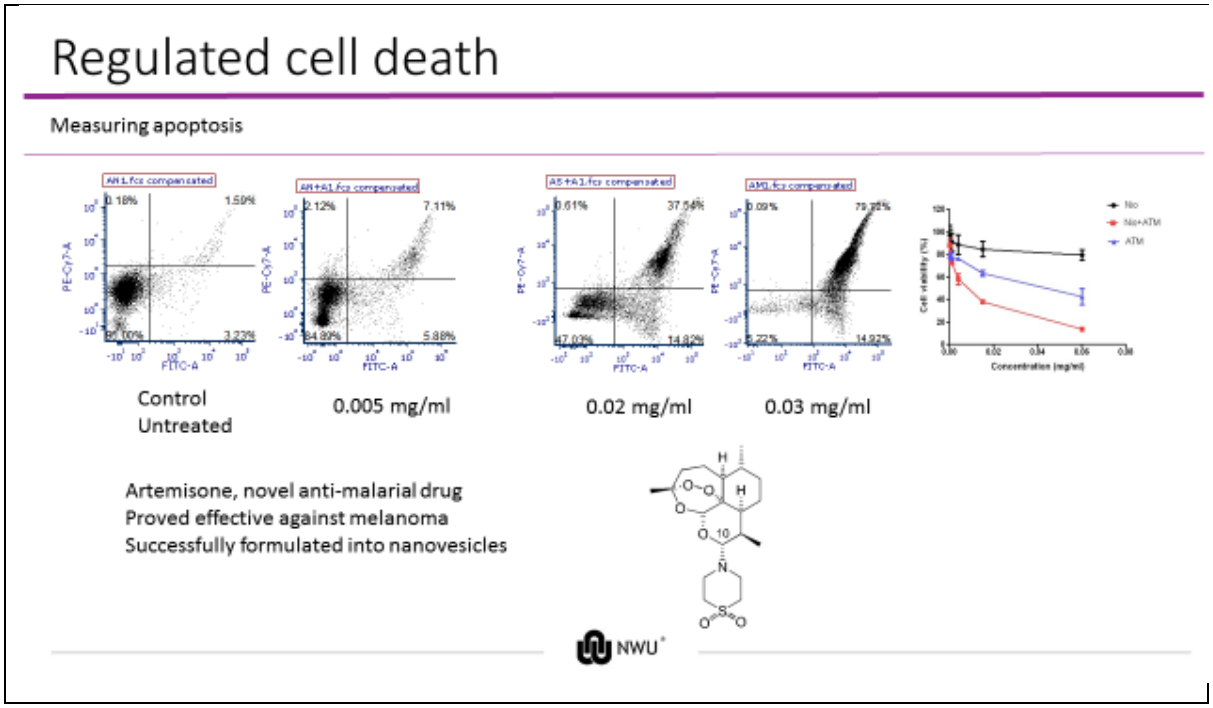



Figure 18 An example of how apoptosis measurement was used to prove the anti-cancer effects of artemisone, a novel anti-malarial compound.

**Conclusion**

Although microscopy remains the gold standard in cytometry we have proven the value of using flow cytometry in the study of how cells die. It offers advantages including rapid, mulitparameter analysis of large populations of individual cells, that microscopy cannot offer. I have shown that the process of cell death may seem contradictory with physiological and pathological mechanisms. But we can also use these contradictions to our advantage in developing new drugs or novel dosage forms. Not only were we able to characterise different types of cell deaths, I also worked on many other applications with flow cytometry. In total since its conception at the NWU flow cytometry has contributed to the training of 35 MSc students in various disciplines including biochemistry, physiology, chemical engineering, veterinary sciences, pharmaceutics, pharmaceutical chemistry and pharmaceutical sciences. Additionally 12 PhD's and five post-doctoral fellows used flow cytometry in their studies. A total of 15 publications and 36 conference contributions where I was directly involved in was published or delivered. In the 11 years there was also collaborations with 11 different departments at the NWU and other Universities or research institutions.

## Future directions

Currently I was given the opportunity by Prof Deon de Beer to start working in the additive manufacturing field and more specifically, 3D bioprinting. With 3D bioprinting cells are laid down layer-by-layer moving from monolayer cell models to multilayer models. This novel development we could move from simple cellular systems for investigating how cells die, to more complex, 3D physiologically relevant cellular models. Rather than looking at the effects of only one or to cell types, these models will mimic whole organs or organ systems and further help us understand How cells die. Who knows, if Mark Fulwyler could combine cell analysis with inkjet printing, maybe scientists will be able to develop an all in one 3D printing and cytometry technology in the future.

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