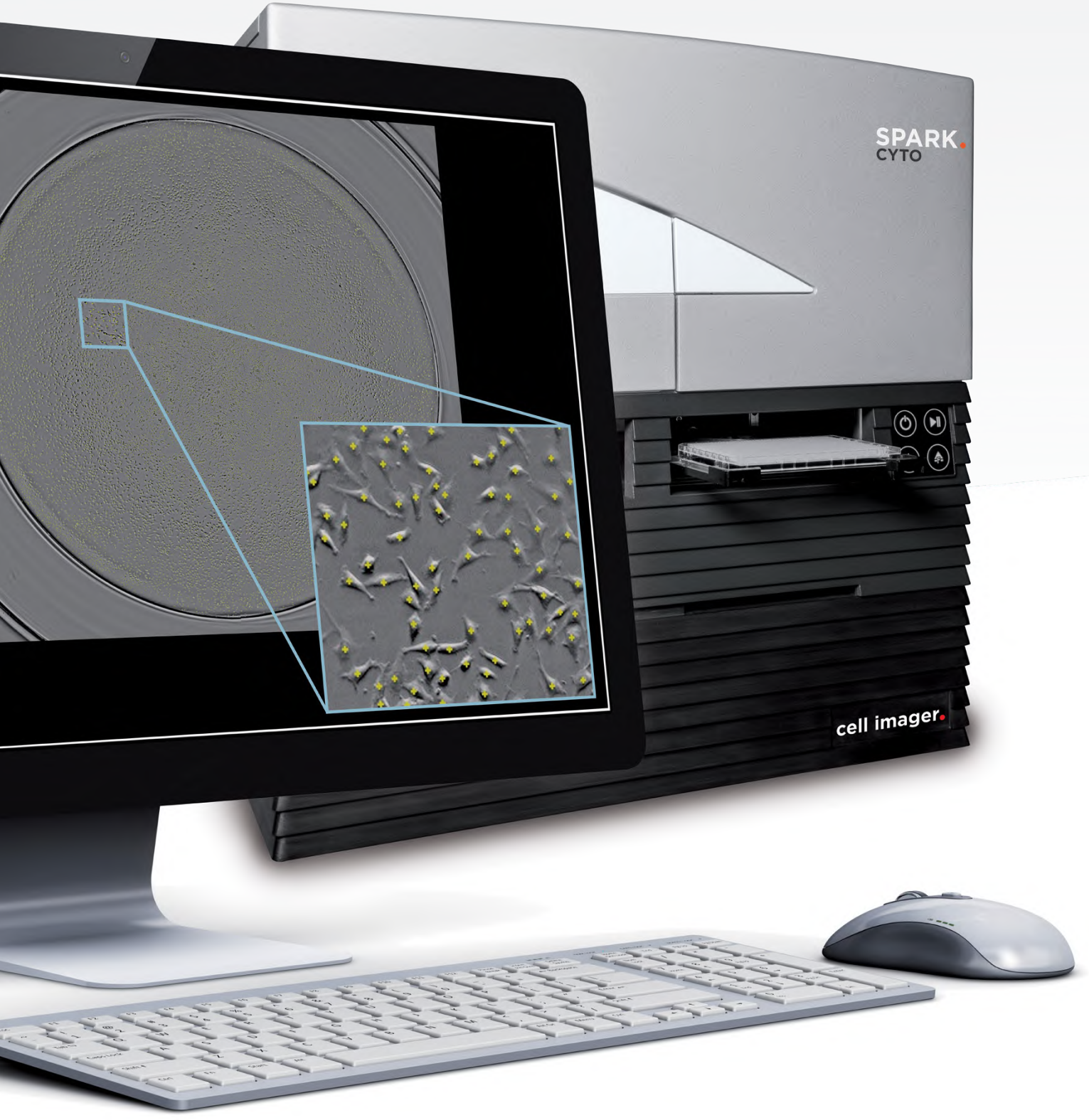
APPLICATION GUIDE FOR MULTIMODE READERS.



TECAN.

TECHNOLOGY ORIENTATION.

Cell imaging	**4**
Absorbance	**20**
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Time-Resolved Fluorescence	**38**
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Fluorescence Polarization	**52**
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CELL IMAGING.

Imaging-based analysis of cell samples using Spark® and Spark Cyto.

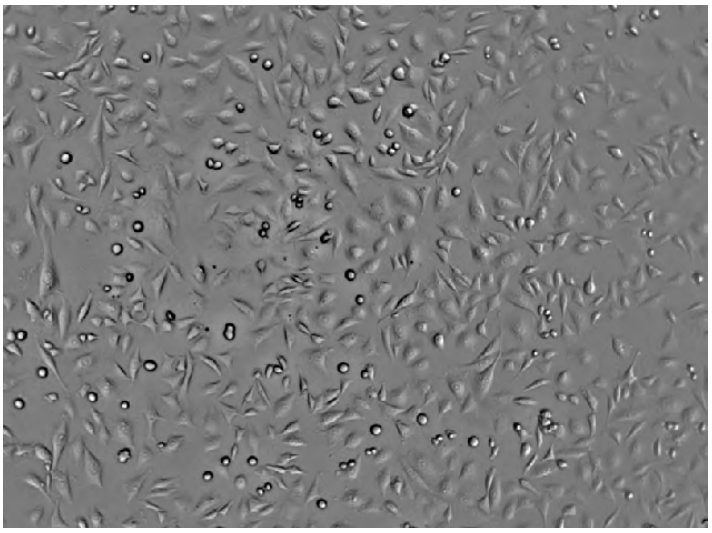


Figure 1A: Bright field cell imaging of a CHO cell with digital zoom applied, using the Spark.

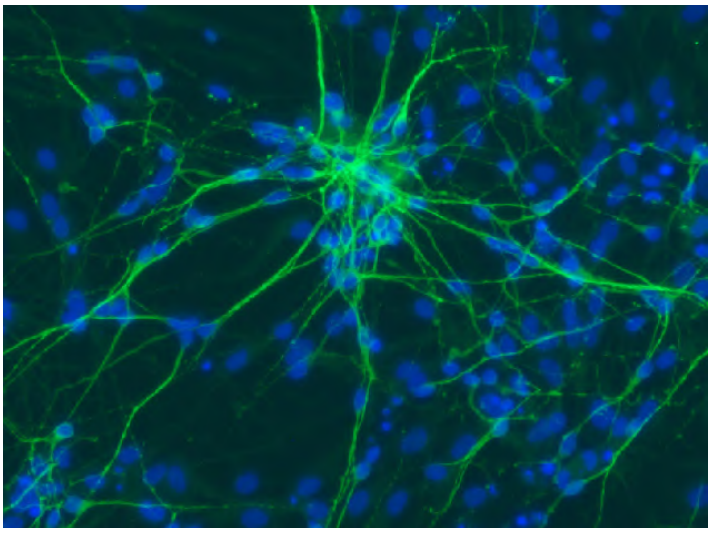


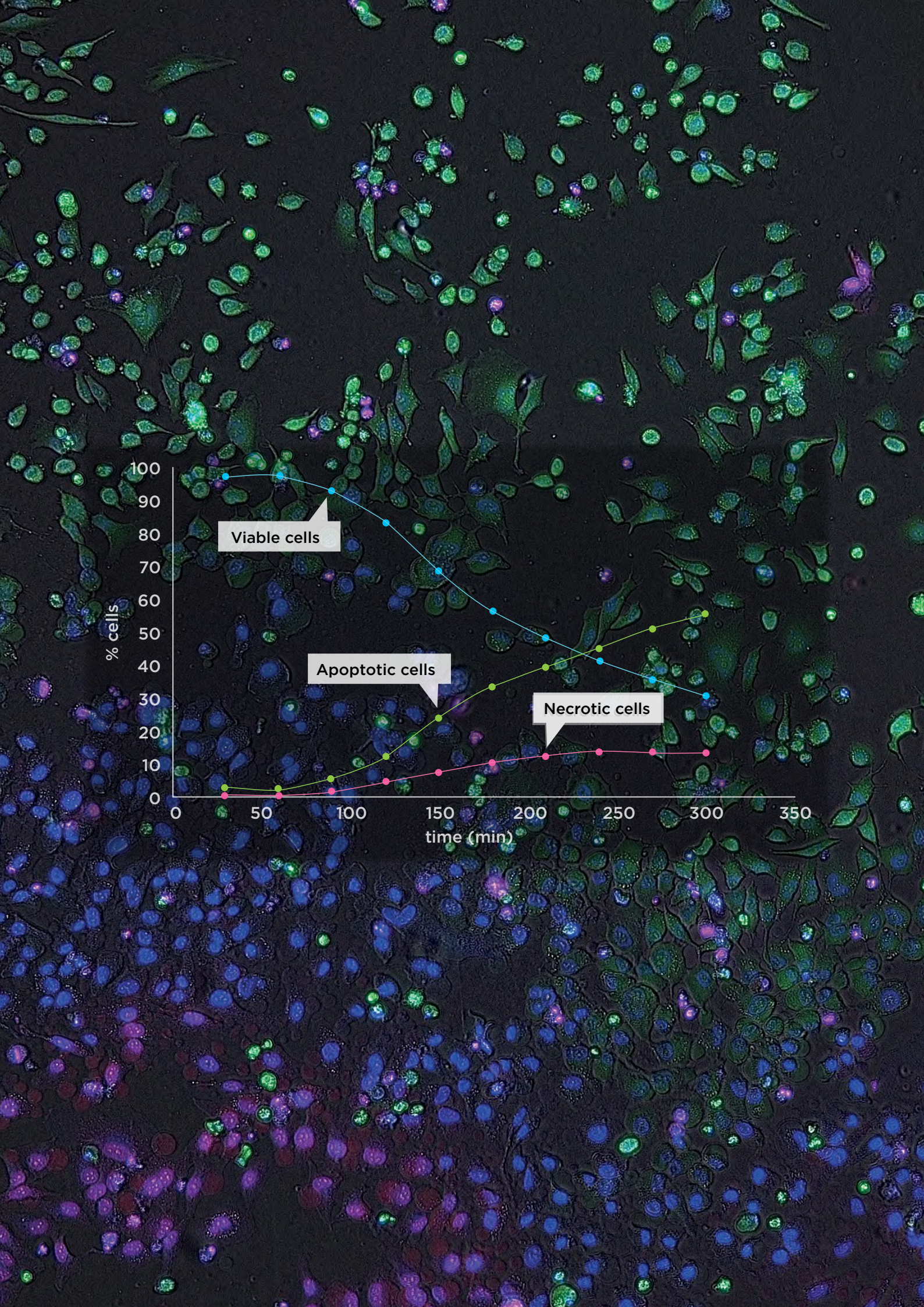
Figure 1B: Human Neuronal Cells in a 24-well plate stained with MAP2 (green) and DAPI (blue) imaged with the 10x objective on the Spark Cyto.

TECHNOLOGY.

Cell-based assays in microplate formats have become a vital part of life science experiments, with cell growth, proliferation, migration, and cytotoxicity being central research objectives. However, confluence levels, gene expression activity, and the mode of cell death are also important parameters that are routinely analyzed in cell culture laboratories. In this context, imaging-based readouts offer valuable insights and provide the basis for reproducible analysis and accurate interpretation of experimental data. Automating and standardizing these processes streamline cell-based workflows and help to free up time in the lab.

Tecan's Spark multimode reader has a cost-effective, built-in cell imaging module, enabling automated cell counting and viability analysis in slides (Tecan's cell chips), as well as bright field imaging and automated confluence assessment in microplates (see Figure 1A). Spark's imaging module includes LED bright field illumination, 4x objective magnification with an optical resolution of greater than or equal to 3 µm, a 1.3-megapixel CMOS camera, and a robust autofocus system. It offers full environmental control, including temperature, CO_2, and O_2, as well as evaporation protection. Injectors with reagent heaters and stirrers, and integrated microplate lid-handling are also available. With these features, Spark delivers better quality and more physiologically relevant data for your cell-based research and opens the door to the exciting world of live cell imaging.

The Spark Cyto is the first multi-mode plate reader with live cell imaging and real-time cytometry capabilities. It contains the same detection modes, features, and functions as the Spark but is also equipped with a high-performance bright field and fluorescence imaging module (see Figure 1B), unlocking new possibilities for your cell-based research. Spark Cyto uniquely brings together top-of-the-range camera components with proprietary, patent-pending technology to ensure that you can truly investigate your entire cell population. It gives you the ability to record the whole well area of a 96- or 384-well microplate with just one image, without tiling or distortion. This means that you never miss a cell when investigating the total population in a microplate well. Spark Cyto includes three magnification levels, combined with four channels for fluorescence and bright field imaging, enabling high-quality cell analysis for a wide variety of applications. The system is controlled by the versatile and user-friendly SparkControl software, offering both ease of use and flexibility for operators. Images acquired with the Spark Cyto can be automatically processed with Image Analyzer, Tecan's proprietary imaging software package. Image Analyzer offers you an array of customization options, making it easy to adjust and optimize your image analysis parameters.


100
90
80
70
60
50
40
30
20
10
0
% cells
Viable cells
Apoptotic cells
Necrotic cells
0
50
100
150
200
250
300
350
time (min)

Reader	Spark	Spark Cyto
Features		
Imaging modes	Bright field	Bright field, Digital phase Fluorescence
Sample formats (for imaging)	6 to 96-well	6 to 384-well
Camera sensor	CMOS, 1.3 megapixel	CMOS, 5.0 megapixel
Objectives	4x	2x, 4x, 10x
Max. optical resolution	3.0 µm	1.2 µm
Channels	Bright field	Bright field Four fluorescence channels (blue, green, red, far-red)
Autofocus	LED	Astigmatism-based LED, image-based
Field of view	Center User-defined Whole-well by tiling	Center User-defined Whole-well, 96- and 384-well with single image Whole-well by tiling, 6-48-well plates
Image Analysis	Real-time analysis; speed: <45 min for 96-well (whole-well by tiling) + confluence assessment	Real-time analysis; speed: ≤20 min for 96-well (whole-well by single image) + confluence assessment
Re-analysis capability	N/A.	Image Analyzer software

Analysis	Spark	Spark Cyto	Key applications
Counting in slides	●		Suspension / trypsinized cell counting and viability (Trypan Blue)
Area Brightfield	●	●	Confluence, proliferation, cytotoxicity, wound healing, transfection efficiency, viral infection ...
Fluorescence		●	
Counting Brightfield		●	Cell viability, transfection efficiency, proliferation, cytotoxicity, GFP reporter ...
Fluorescence		●	
Multi-Color		●	Apoptosis, necrosis, bacterial infection ...
3D		●	Spheroid and organoid proliferation, viability, cytotoxicity ...

SUPPORT.

Links.

- https://lifesciences.tecan.com/plate-reader-live-cell-imaging-cytometry
- http://lifesciences.tecan.com/live-cell-imaging
- http://lifesciences.tecan.com/cellbiology

CELL COUNTING AND VIABILITY OF CELLS IN SUSPENSION.

Determine the number and health of your cells using Tecan Cell Chips™.

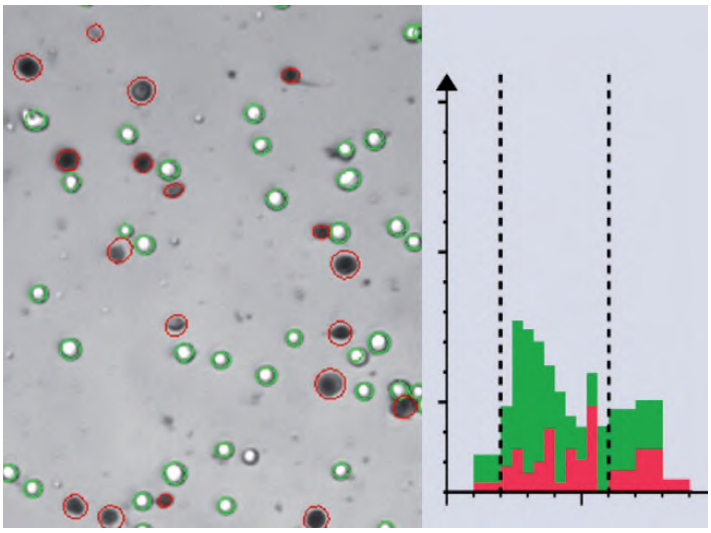


Figure 1 A: Discrimination of live/dead cells in suspension using Spark.

Figure 1 B: Cell Chips on adapter plate.

Technology.

Cell-based assays in a microplate format have become a vital part of life science research; proliferation, cytotoxicity, and gene expression studies all inevitably use cells as a working tool. Regardless of the research area, cell counting and quality checks are commonly performed before cell seeding or passaging steps. One approach is to identify if cells are alive or dead by staining with trypan blue; only dead cells or those with a damaged cell membrane will be stained blue, enabling them to be discriminated from viable, healthy cells (Figure 1A).

The Spark's cell imaging module offers automated, label-free cell counting and trypan blue-based cell viability analysis with an easy-to-use, disposable Cell Chip (Figure 1B). Each Cell Chip has two sample chambers that can accommodate independent cell suspensions or replicates of the same sample. Four Cell Chips can be loaded onto the Cell Chip adapter plate, allowing up to eight samples to be analyzed in less than two minutes.

The cell counting and cell viability applications both offer the same features, with the difference that the viability application automatically takes into account a 1:1 trypan blue dilution when calculating the results, displaying the number and percentage of viable cells. Users can set the cell size to between 4 and 90 µm before starting the measurement, allowing you to tailor the assay to your chosen cell line. For better counting accuracy and reproducibility, the system also allows you to select one, four, or eight images per chamber.

This enables fast cell counting for applications with relatively high cell concentrations (approximately 1 x 10^6 cells/ml) by taking a single image at the center of each chamber. In contrast, for low cell concentrations (approximately 1 x 10^4 cells/ml), eight separate images can be used per sample to dramatically improve the accuracy of cell concentration measurements. Furthermore, the two chambers of each slide can be defined as "duplicates" for automatic evaluation. Cell counting and cell viability results are clearly presented in a table and histogram on the software dashboard, allowing straightforward size distribution analysis of the cell population. All results are then saved in a PDF report for easy and convenient data storage.

MAJOR APPLICATIONS.

- Cell culture quality control
- Cell seeding
- Cell proliferation studies

SUPPORT.

Link.

https://lifesciences.tecan.com/live-cell-imaging?p=tab--2

CELL CONFLUENCE.

Check the growth status of your cells.

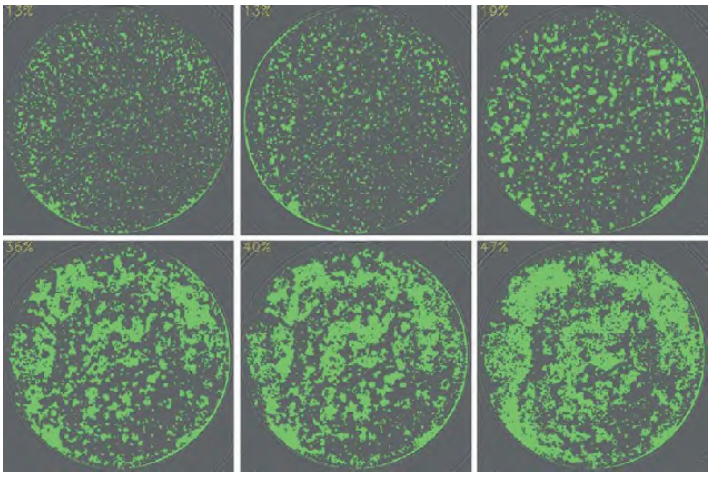


Figure 1A: Continuous growth monitoring assessing the confluence in the Spark (96-well images; tiled)

Technology.

In cell culture applications, confluence assessment is an important quality control parameter. It is commonly used to estimate the proportion of adherent cells on a growth surface as an indicator of the cell density inside a well or culture flask. In addition, the growth rate or gene expression of some cell lines varies depending on the degree of confluence. Consistent determination of cell confluence and estimation of cell numbers are therefore important for reproducible assays and accurate interpretation of experimental data. Image capture and confluence determination for adherent cells directly in microplates improve crucial scientific parameters, like assay variation and cell seeding uniformity.

Spark's cell imaging module allows you to precisely determine the confluence level in a well from 10 to 90%, giving you a new level of data quality for your assays. The easy-to-use SparkControl software allows you to select different imaging areas within the well. Depending on the assay, you can either select just a single image of the well center or a full-well picture composed of multiple images, with automatic well border detection to compensate for variations in microplate dimensions.

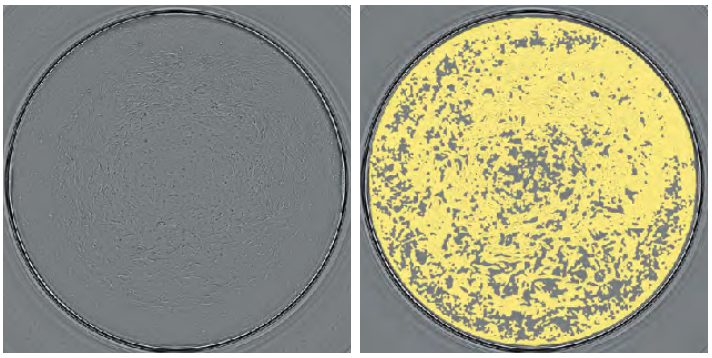


Figure 1B: Confluence in the Spark Cyto: 96-well plate imaged with unique whole-well imaging using the 2x objective and analyzed with the confluence algorithm. Processed bright field image (left) and applied confluence mask depicted (right). No tiling or edge-to-edge optical distortion leads to superior results when analyzing cell populations in terms of confluence.

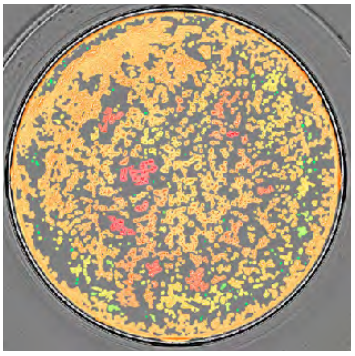


Figure 1C: The roughness factor is a qualitative assessment of the cells' granularity.

Confluence is assessed in real time, with data displayed on the dashboard and exported directly to Excel for further analysis. Each confluence value is linked directly to the raw and analyzed images, enabling quick and easy visual confirmation of cell confluence.

Confluence determination is completely independent of cell morphology, using an algorithm designed to offer exceptional sensitivity, even when working with low-contrast cells, such as fibroblasts. This makes Spark's confluence measurement compatible with virtually any cell type, offering an easy-to-use approach to improve your assays' uniformity. Label-free confluence assessment can also be used as a replacement for costly and time-consuming cell mass analyses like the quantification of protein levels or ATP content. It also enables applications like cell migration, wound healing, and clonogenicity assays.

Spark Cyto allows you to image a whole well in a 96- or 384-well plate with a single picture using proprietary, patented high-dynamic-range (HDR) imaging technology. This allows you to record the complete picture of your cell population in less time, enabling you to drive your research in new directions. Re-analysis and analysis adjustment capabilities allow the user to adapt the sensitivity of the confluence assessment according to the experimental conditions. An extremely useful feature of the Spark Cyto is the calculation of the roughness factor within confluence assessment. This is a qualitative assessment of the cells' granularity and is expressed as the normalized mean standard deviation of pixel intensities over all separated areas. It provides a simple measure of the relative signal variation across the cell population and can indicate cytotoxicity, cell division, and even the level of bacterial or yeast contamination without the need for fluorescent staining.

The dimensionless roughness factor can have a value between zero and infinity. The mask ranges from green to red depending on the normalized mean standard deviation of pixel intensities over all separated areas, which represents the granularity of the cells. It has been shown that this automated, non-invasive readout allows for high-throughput studies of virus infection. An increased roughness factor was observed due to the formation of syncytia and cell lysis induced by the infecting virus.

MAJOR APPLICATIONS.

- Cell culture quality control
- Cell migration and wound healing studies
- Cell proliferation studies
- Cytotoxicity studies

SUPPORT.

Links.

- https://lifesciences.tecan.com/plate-reader-live-cell-imaging-cytometry?p=tab--3
- https://lifesciences.tecan.com/live-cell-imaging?p=tab--3

BRIGHTFIELD CELL COUNTING OF ADHERENT CELLS IN MICROPLATE WELLS.

Label-free cell count using an AI-based analysis.

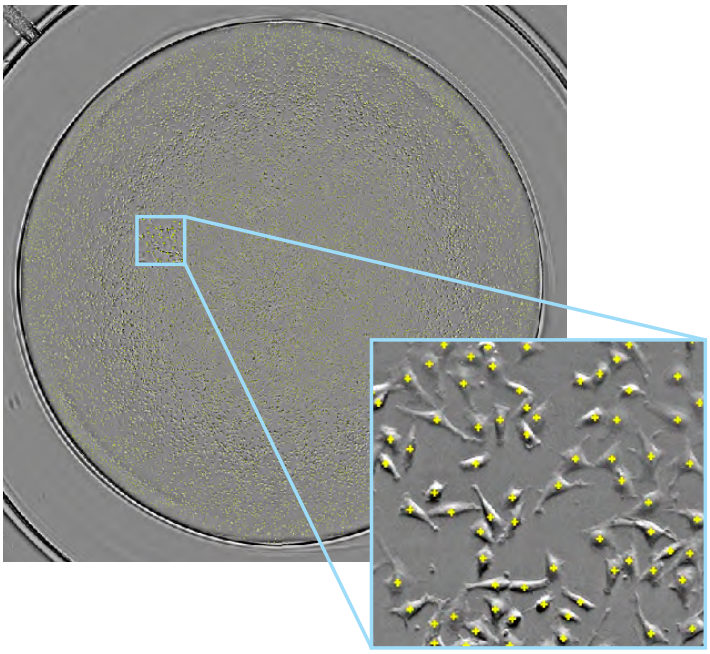


Figure 1: Label-free cell count of a whole 96-well in 4x objective. Zoomed in image shows the counting mask based on a AI based analysis.

Technology.

Assessing the cell number to monitor growth per well is an inevitable procedure across a wide range of cell biological applications. However, conventional methods can impact cellular behavior and are not suitable for kinetic monitoring of the cell population. The assays either rely on fluorescent dyes (DAPI, Hoechst 33342) to determine the cell count or measure the metabolic activity (MTT, ATP) and correlate it to the number of cells present. Both interfere with the sample and prevent further downstream analysis. Therefore, there is a significant need for label-free cell counting without any perturbation.

The Spark Cyto offers a label-free counting approach in adherent cells in a well plate. This non-invasive feature is an alternative to conventional nuclei counting avoiding potential cytotoxic side effects. The identification of the cells in brightfield is performed using artifical intelligence, where a deep learning algorithm was developed using images of different, commonly used cell lines with varying morphologies. The number of cells in a whole microplate well are quickly and reliably assessed in an automated way. Results are immediately displayed in real time after image acquisition and no further analysis step is required. The label-free object mask can be checked in the Image Analyzer™ software of the Spark Cyto, to visually verify the performance of the algorithm. This noninvasive approach can bring benefits to cell-based workflows in any laboratory reducing costs for additional reagents and saving time with no extra preparation steps.

MAJOR APPLICATIONS.

- Cell culture quality control
- Cell proliferation studies
- Normalization to cell number per well for all cell-based assays measured with standard readouts (e.g. luminescence signals)

SUPPORT.

Links.

- https://lifesciences.tecan.com/plate-reader-live-cell-imaging-cytometry?p=tab--

NUCLEI COUNTING.

Accurately determine the number of cells in a microplate well.

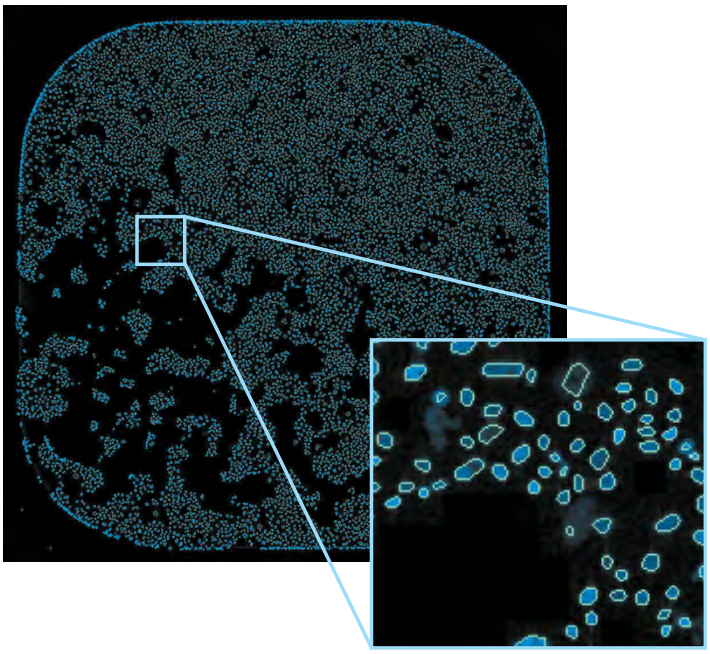


Figure 1: Whole-well image of cells stained with Hoechst 33342, using a nuclei counting mask .

Technology.

Cell counting is a widespread measurement in tissue culture to assess density for cell-based assays, as a readout in proliferation, viability, and toxicity assays, and to normalize cell-based assay results. Cell counting and viability assessment of suspension cells in slides is available with the Spark, but Spark Cyto offers imaging of adherent cells cultured in a monolayer using fluorometric probes that intercalate with nuclear DNA. This function provides an easy method using any dye with nuclear binding capabilities to count cells independently of cell morphology. The ability to perform whole-well imaging of 96- or 384-well plates with a single picture ensures accurate cell counting and always reveals the full picture. Images and results can even be re-analyzed and optimized using the Image Analyzer.

MAJOR APPLICATIONS.

- Cell culture quality control
- Cell proliferation studies
- Cytotoxicity studies

SUPPORT.

Links.

- https://lifesciences.tecan.com/plate-reader-live-cell-imaging-cytometry?p=tab--3
- https://lifesciences.tecan.com/live-cell-imaging?p=tab--3

TRANSFECTION EFFICIENCY.

Transfection efficiency checks and quantification of protein expression in microplates.

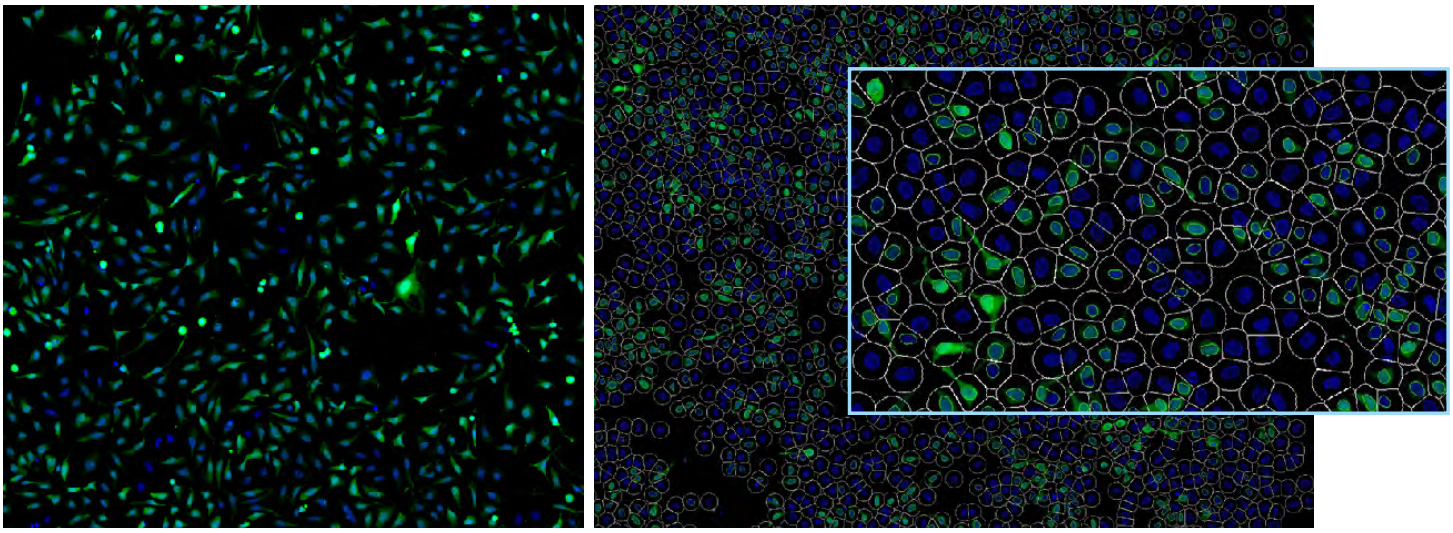


Figure 1: HeLa GFP cells grown in a 96-well plate and stained with a nuclei marker (Hoechst 33342) to determine the transfection efficiency. Images were taken with the 10x objective of the Spark Cyto and the Multicolor mask is shown on the right image.

Technology.

Transfection and transduction are indispensable tools for studying biological functions and processes. Assessing transfection/transduction efficiency of cells in a qualitative and quantitative manner is frequent and important for many laboratories to ensure that cell samples are suitable for downstream applications. Transfection efficiency (TE%) is usually assessed with the aid of fluorescent labels, such as green fluorescent protein (GFP), either manually by fluorescence microscopy or using single-cell analysis techniques such as flow cytometry. However, this requires detachment of the cells from the plates. Live cell imaging permits the analysis of cell expression *in situ.*

An easy and fast determination of transfection efficiency can be performed using the Spark Cyto imaging module. Without any preparation procedure or detachment of the cells, the transfection efficiency can be assessed in living cells. Cells are imaged in bright field and, e.g., the green fluorescent channel if transfected with GFP. The SparkControl performs immediate calculations to correlate the label-free cell count in bright field with the fluorescent signal, thereby calculating the transfection efficiency and displaying it in real time.

An even more accurate determination of transfection efficiency can be performed using the multicolor application of the Spark Cyto. However, this requires an additional preparation step to perform a nuclei stain. The algorithm first segments the nuclei (primary mask), followed by detection of a signal within the analysis area previously defined by the user (secondary mask). The signals are automatically correlated, and the transfection efficiency is shown.

MAJOR APPLICATIONS.

- Transfection and transduction efficiency
- Reporter assays

SUPPORT.

Links.

- https://lifesciences.tecan.com/plate-reader-live-cell-imaging-cytometry?p=tab--3
- http://lifesciences.tecan.com/cellbiology

CELL VIABILITY.

Quantification of live and dead cells in microplates.

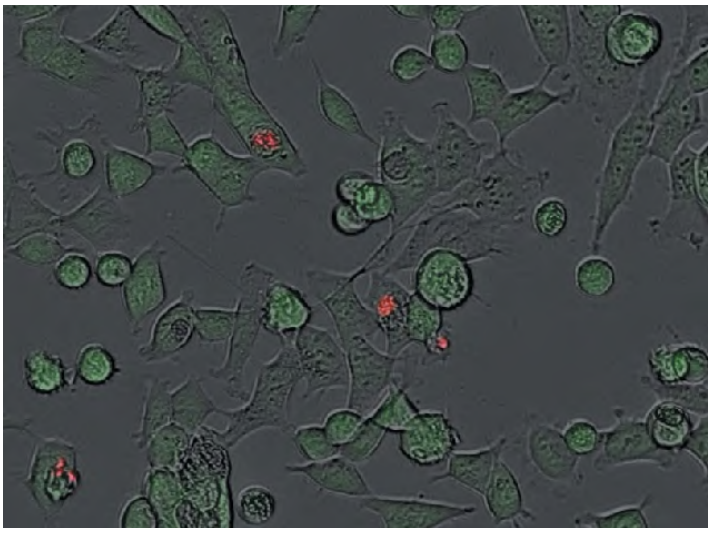


Figure 1: HeLa cells cultured in a 24-well plate, acquired with the 10x objective, showing an overlay of the bright field, green and red channels (digital zoom applied).

Technology.

Viability assays are commonly used in cell biology labs as a screening tool to assess cell line response to compounds of interest or different growth conditions. There are numerous methods for determining cell viability, some providing "well" information – e.g., bioluminescent detection of ATP or colorimetric assays using MTT, WST-1, or resazurin – while others use fluorescence-based imaging to discriminate between live and dead cells on an individual level. These fluorescent viability markers are usually combined with appropriate cell death markers, based on membrane permeability and accessibility of the nuclei.

Using the Spark Cyto, cell viability can be assessed by performing a double staining to discriminate between live and dead cells in a population. The standard counting algorithm is applied to count living and dead cells and correlate them to calculate the cell viability automatically.

An assay example is using Calcein-AM (live cells) and propidium iodide (dead cells) to image and analyze the cell population in minutes. Calcein-AM is a non-fluorescent, cell-permeable derivative of calcein that becomes fluorescent upon hydrolysis within the cytosol. Propidium iodide (PI) is a fluorescent intercalating dye that binds to DNA with little or no sequence preference. It is not membrane permeable, making it useful to differentiate necrotic, apoptotic, and healthy cells based on membrane integrity.

MAJOR APPLICATIONS.

- Viability assessments
- Cytotoxicity studies

SUPPORT.

Links.

- https://lifesciences.tecan.com/plate-reader-live-cell-imaging-cytometry?p=tab--3
- http://lifesciences.tecan.com/cellbiology

CELL DEATH.

Detecting apoptosis of adherent cells in microplates.

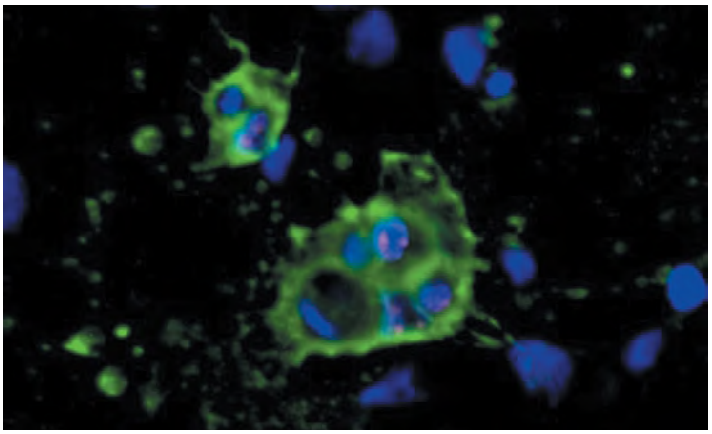


Figure 1A: Apoptotic A431 cell imaged with the 10x objective and digitally zoomed-in.

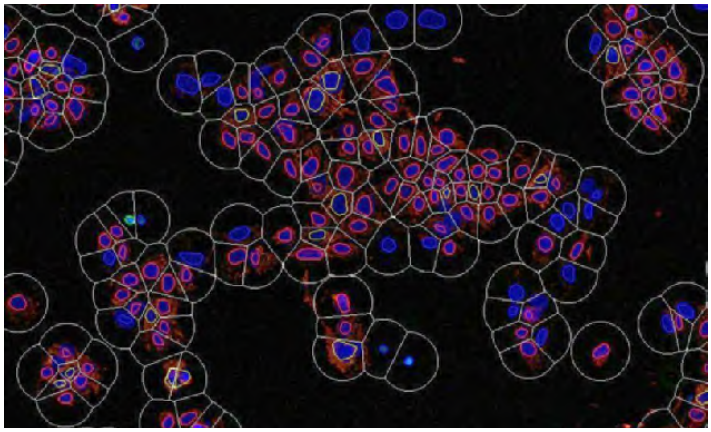


Figure 1B: HepG2 cells grown in a 384 well plate and imaged with the 10x objective on the Spark Cyto to quantify apoptosis based on a triple-staining. The MultiColor algorithm segmentation mask is shown.

Technology.

Apoptosis, or programmed cell death, is a central component of many biological processes, including development, tissue homeostasis, and the maturity and maintenance of the immune system. The identification and discrimination of apoptotic, necrotic, and viable cells is therefore important for a variety of biological research fields. The detection of, and discrimination between, viable, apoptotic, and necrotic cells can be accomplished by differential staining with specific markers for viability, apoptosis, and necrosis. The multicolor algorithm of the Spark Cyto is typically used to perform this subpopulation analysis. It correlates multiple fluorescence signals within a single object by using up to three fluorescence channels. The feature requires a nuclear marker or staining to first segment the cell nuclei (primary mask, nuclear mask). Next, one or two more fluorescence signals can be detected in the analysis area previously defined by the user (secondary mask). This results in correlating the object count (primary mask) with signals found in the secondary mask. Using three fluorescent dyes, you can image and analyze your cell population for apoptotic events within minutes. A good example of an assay used to detect apoptosis is the Annexin V stain (FITC-labeled) in combination with a nuclear dye (Hoechst) and a necrosis marker (PI). In healthy cells, phosphatidylserine (PS) is located on the inner leaf of the plasma membrane.

As one of the first steps in apoptosis, PS is externalized, which is a signal for the cells to be phagocytized. Once present on the outer leaf, it can be detected by the calcium-dependent protein annexin V conjugated to a fluorescent label, such as fluorescein isothiocyanate (FITC) or Alexa Fluor 488. Adding a second, cell-impermeable dye, such as PI, permits the discrimination of necrotic cells, because only these cells have permeabilized outer membranes, making them accessible to the dye. Staining with PI and FITC/Alexa Fluor 488 is a standard procedure to discriminate apoptosis and necrosis in cell cultures; early apoptotic cells are annexin V positive and PI negative, whereas late apoptotic cells are annexin V/PI double positive, as shown in Figure 1A. The total number of cells can then be identified by staining with a nuclear dye, such as Hoechst 33342 or DAPI..

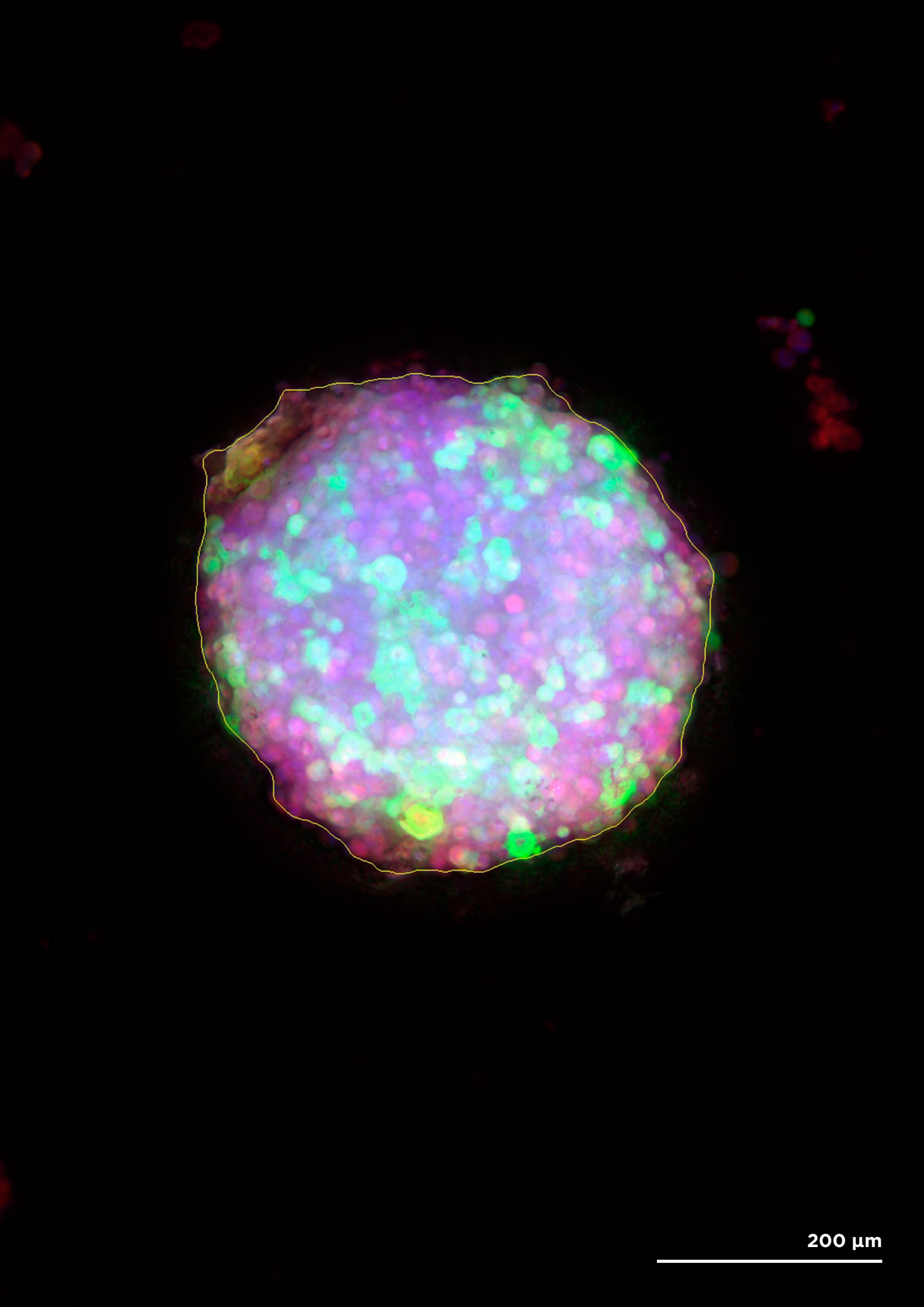
MAJOR APPLICATIONS.

- Cell death discrimination
- Apoptosis quantification
- Necrosis quantification

SUPPORT.

Links.

- https://lifesciences.tecan.com/plate-reader-live-cell-imaging-cytometry?p=tab--3
- http://lifesciences.tecan.com/cellbiology


200 µm

IMAGING OF 3D CELL CULTURE MODELS.

Performing Imaging in flat and round bottom plates, automated z-stacking and AI-based image analysis.

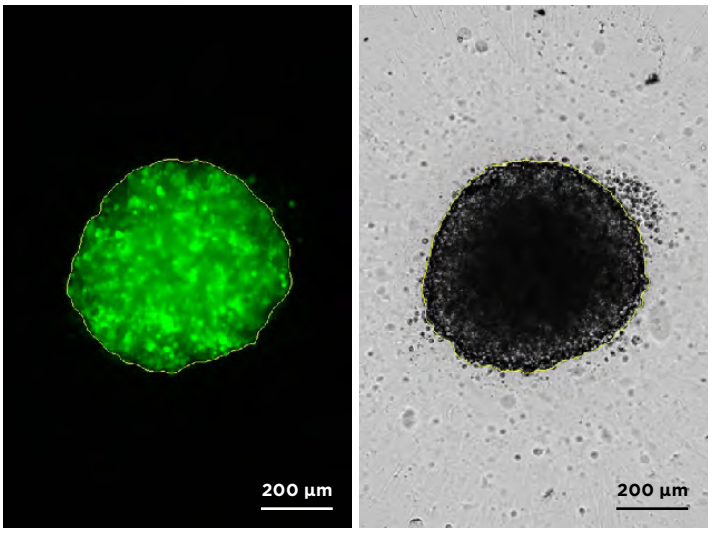


Figure 1A: Single HeLa GFP spheroid grown in a 96 well round bottom microplate. The segmentation can be performed on brightfield or any flourescent channel. A single shot image was aquired with the 4x objective on the Spark Cyto.

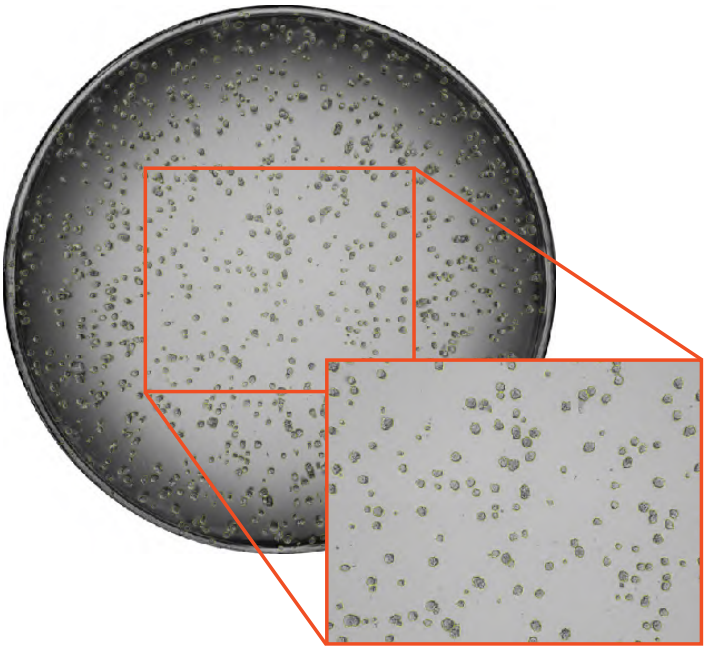


Figure 1B: Whole well image of a 96-well microplate with close-up section on the side. Z-stack imaging of A549 spheroids was performed with the Spark Cyto displaying the projected image in BF channel using the 4x objective.

3D cell culture models more accurately recapitulate the in vivo conditions of native tissues, enabling cells to interact with their microenvironment. Therefore, they show a higher physiological relevance in terms of proliferation, differentiation, metabolism, and gene expression. With this potential, several models have been developed in tissue engineering, drug development, and basic research.

The Spark Cyto is capable of monitoring and analyzing 3D cell models, such as spheroids and organoids, in a label-free manner in bright field, as well as performing cytotoxicity studies and live-dead assays with fluorescent stains. Kinetic monitoring of live cell experiments can be set up to follow growth and track key parameters over time in a controlled environment. The data of a whole microplate is displayed in real time.

One major benefit of the Spark Cyto is the ability to perform multiplexed experiments. Information is not only gathered through imaging but can also be combined with standard “wellular” readouts in luminescence, absorbance, or fluorescence-based assays. This facilitates the generation of multi-parameter data sets in whole-well analysis.

One possible workflow that incorporates the Spark Cyto is shown in Figure 2. It includes cell seeding followed by monitoring of development. The cultured 3D models (spheroids or organoids) are subsequently exposed to drugs, and automated analytical readout is performed. Monitoring of spheroid and organoid growth, as well as imaging and performing standard readouts, such as luminescence or fluorescence assays, with subsequent analysis, can be executed using the Spark Cyto.

Technology.

Imaging of single objects grown in a 3D environment.

One common experimental approach is to grow a single 3D object per well. Therefore, cells are typically seeded in cell-repellent plates and subsequently develop into a spheroid or organoid over time. Spark Cyto 3D imaging is compatible with certain plate geometries, including round-bottom plates. Due to whole-well imaging capability and automated imaging-based autofocus, the whole 3D structure can be captured and automatically analyzed by the AI-based algorithm, either

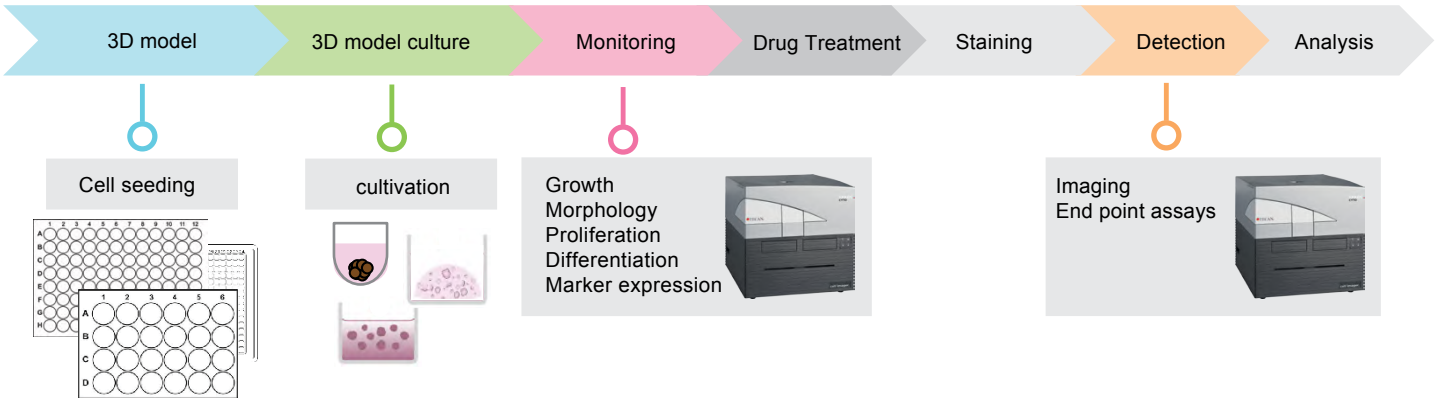


Figure 2: Possible 3D model workflow.

segmented in a label-free manner in bright field or segmented based on a fluorescent signal. The output parameters for single objects are eccentricity, area, x- and y-dimensions, and fluorescence intensities. Usually, a single-shot image for a quick and reliable readout is sufficient to save on time and storage space. However, the z-stacking function with automated generation of a projected image is also applicable in order to capture as much information as possible.

Imaging of multiple objects grown in a 3D environment.

Multiple spheroid or organoid cultures can be obtained when cells are embedded within a matrix and allowed to grow. Due to the matrix, objects are formed on different z-levels. Therefore, the z-stacking feature of the Spark Cyto is fundamental to properly capture all the objects and gather all information present. Subsequently, the z-stack images are processed, and a total projected image is generated, compiling all in-focus information.

The projected images are then used for AI-based analysis of multiple objects. The segmentation is either performed in bright field in a label-free manner or based on a fluorescent signal, which allows for experimental flexibility. The segmentation mask can eventually be changed in the Image Analyzer software and recalculated if necessary. Possible readout parameters for multiple objects include the object count, as well as reporting area, dimension, eccentricity, and fluorescence intensities per object.

MAJOR APPLICATIONS.

- Kinetic monitoring/quality control for growth and morphology
- Proliferation studies
- Differentiation studies
- Drug response
- Dose and time response curve

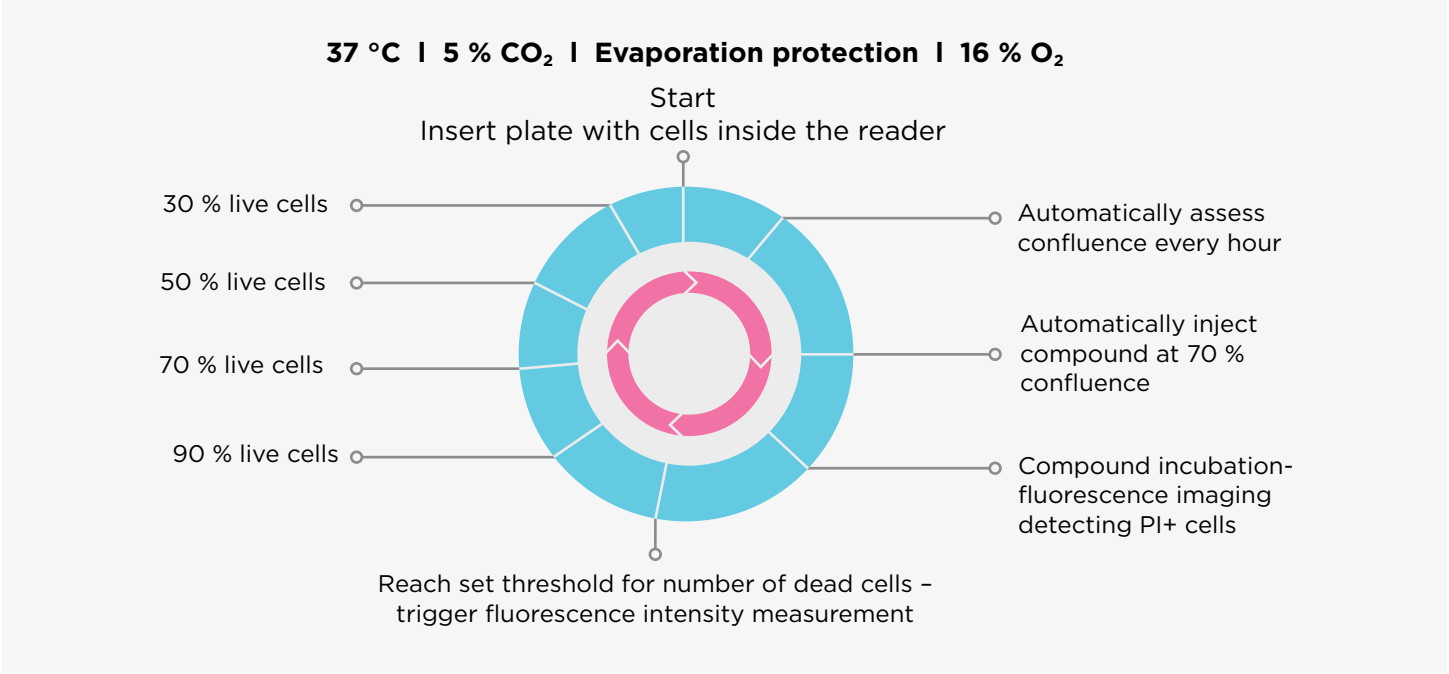
SUPPORT.

Links.

- https://lifesciences.tecan.com/plate-reader-live-cell-imaging-cytometry?p=tab--8

REAL TIME EXPERIMENTAL CONTROL (REC).

Never miss a critical biological event!



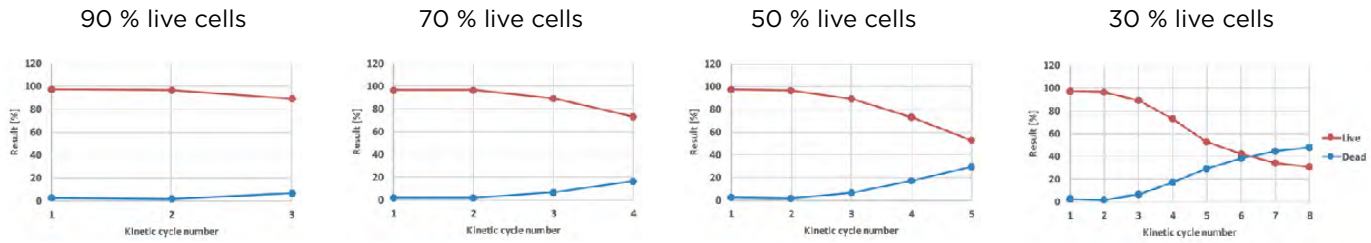


Figure 1: Schematic overview of an exemplified REC case.

Technology.

Many live cell kinetic experiments require specific actions at different points in the experiment – for example, the addition of a compound once a certain confluence or cell count is reached. Real Time Experimental Control (REC) allows certain actions, such as the injection of reagents, to be triggered automatically as part of a kinetic experiment and offers real-time data and image analysis for complete confidence in your results. REC grants you the ability to create new experimental workflows in your lab. By combining standard detection technologies, imaging capabilities, and additional unique features such as integrated evaporation protection and environmental controls, REC unlocks new research possibilities.

REC uses all of these features to create workflows that ensure you never miss a critical biological event – without chaining you to the bench.

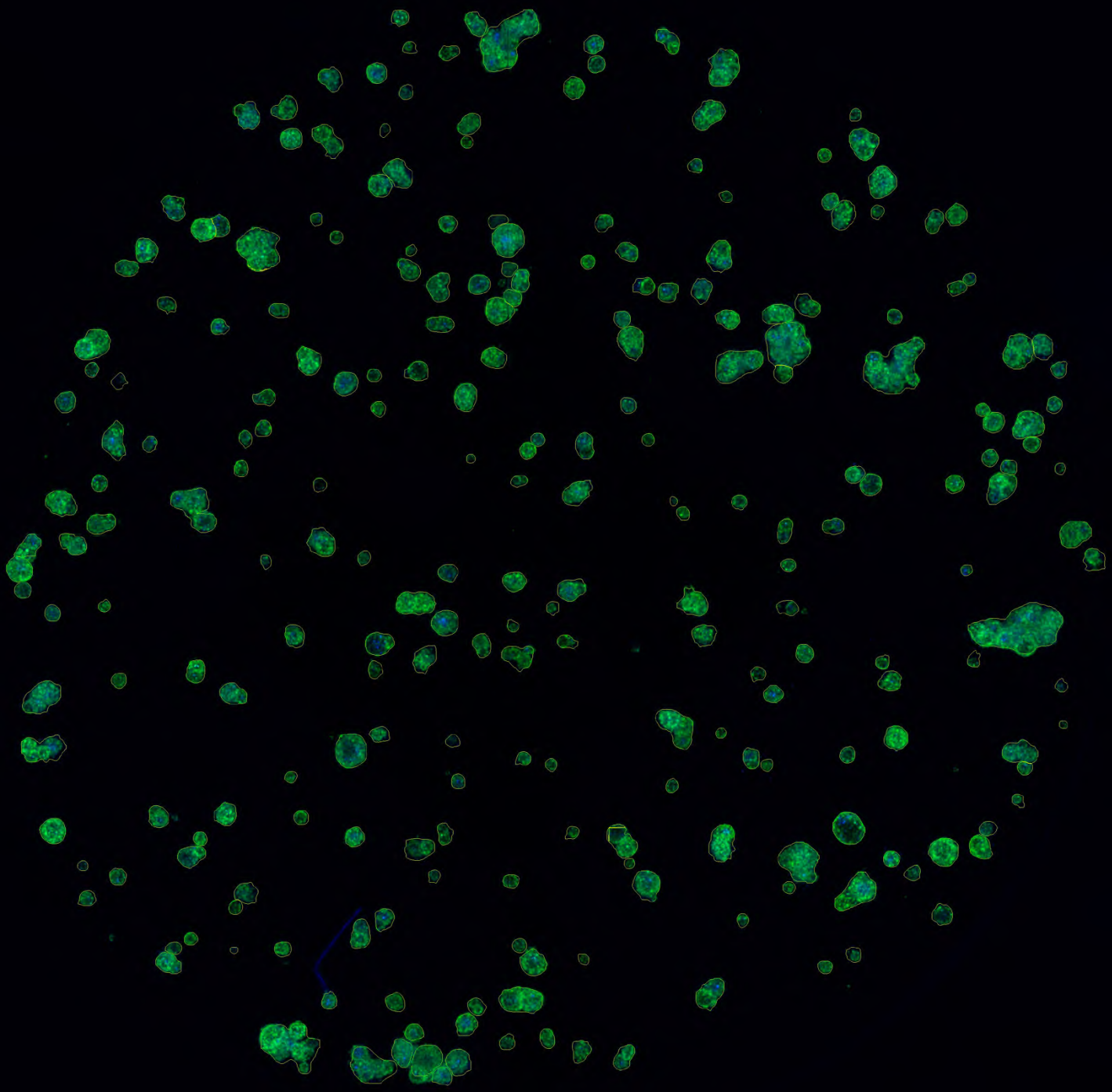
MAJOR APPLICATION.

Cell-based applications in general

SUPPORT.

Links.

- https://lifesciences.tecan.com/plate-reader-live-cell-imaging-cytometry?p=tab--3
- http://lifesciences.tecan.com/cellbiology


200 µm

ABSORBANCE – ABS.

Light is absorbed by the sample.

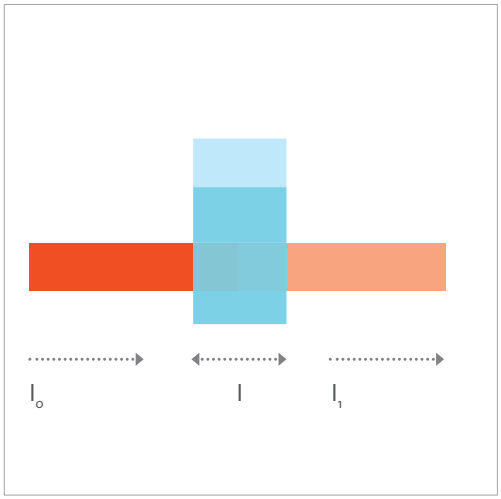


Figure 1: Schematic representation of an absorbance measurement performed in a cuvette.

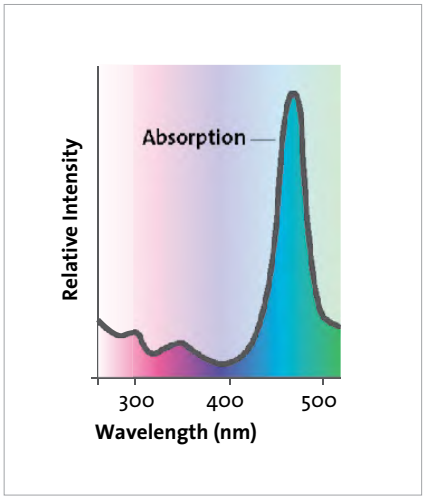


Figure 2: An absorbance spectrum shows the extent of light absorption at any specific wavelength.

Absorbance OD	Transmittance [%]
0	100
1	10
2	1
3	0.1
4	0.01

Table 1: The relationship between absorbance and transmittance values. An absorbance value of 3 means that only 0.1 % of the light is able to pass through the sample. Most multimode readers can only read samples up to an OD of 4.

TECHNOLOGY.

When light shines through a turbid or colored liquid, some of its intensity is absorbed by the liquid's molecules or particles (Figure 1). The amount of light that penetrates the sample and reaches the detector is called the transmittance (T), and the light absorbed by the sample is called the absorbance (A, Abs), or optical density (OD)[1].

OD values commonly correspond to a 1 cm path length, which is the width of standard cuvettes (Figure 1). Note that absorbance is a logarithmic function (Table 1) of the transmittance, as defined by the lambert beer law[2]:

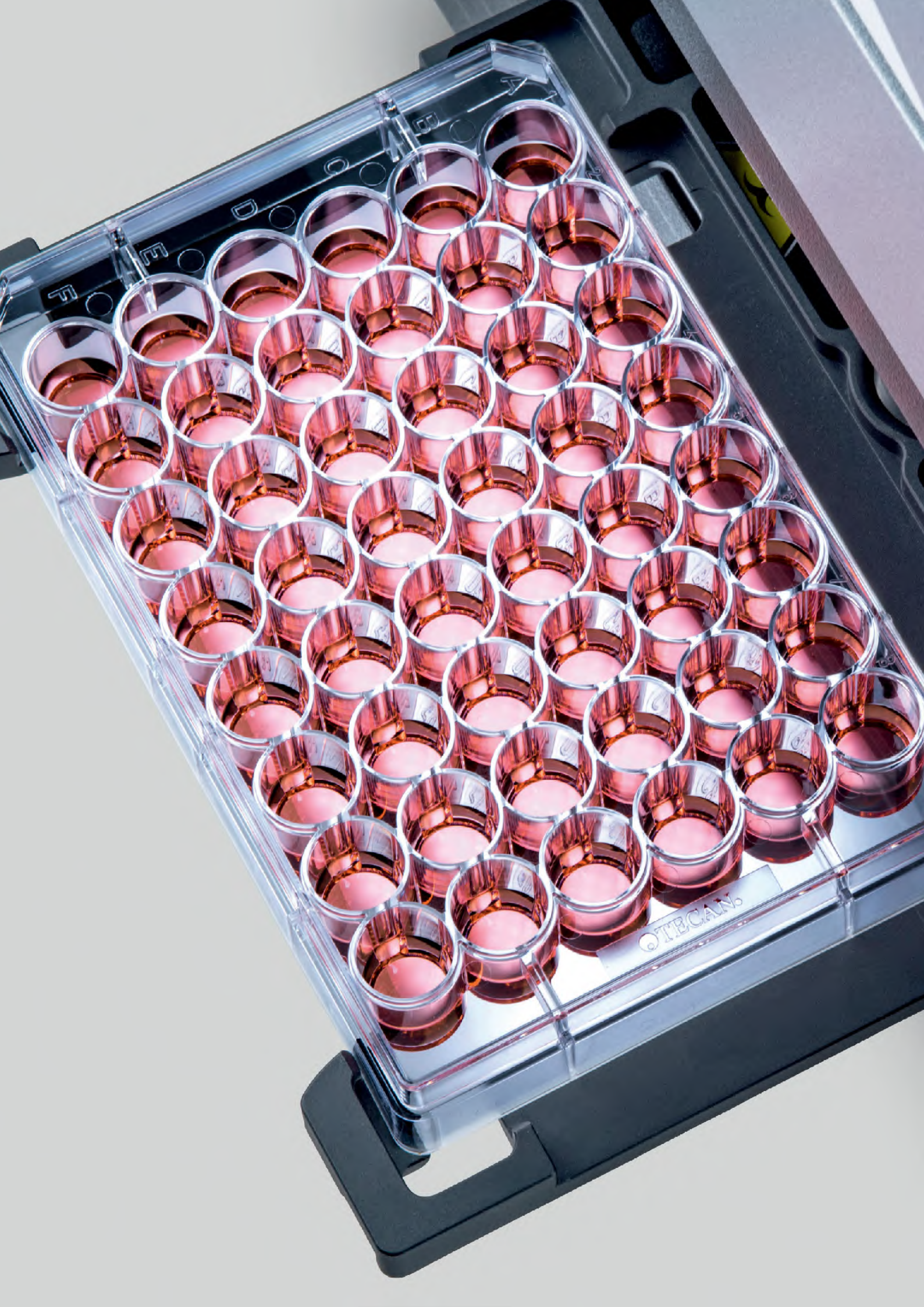
$$A = -\log_{10}(I_1/I_0)$$

The absorbance spectrum is a function of the molecule; light of different wavelengths can be absorbed differently depending on the sample, as shown in the absorbance spectrum in Figure 2. For this reason, the absorbance is always stated together with the wavelength, for example OD_{600}.

Major applications.

- DNA / RNA quantification (Abs)
- MTT / MTS assays
- BCA, Modified Lowry and Bradford assays – Protein quantification

[1] Bioanalytik. Von F. Lottspeich. Spektrum, Heidelberg, 1998
[2] Beer (1852) "Bestimmung der Absorption des rothen Lichts in farbigen Flüssigkeiten" (Determination of the absorption of red light in colored liquids), Annalen der Physik und Chemie, vol. 86, pp. 78–88.


TECAN

DNA / RNA QUANTIFICATION.

DNA / RNA quantification based on UV-absorbance .

Figure 2: Tecan NanoQuant Plate™

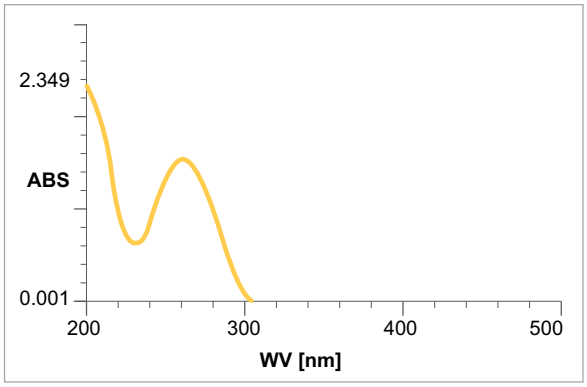


Figure 1: DNA absorbance spectum.

$1OD_{260}$	Concentration (µg/ml)
dsDNA	50
ssDNA	33
RNA	40
Oligonucleotides	20–30

Table 1: Nucleic acid concentrations at OD_{260} = 1 and neutral pH

ASSAY OVERVIEW.

Technology.

Absorbance

DNA and RNA can be quantified based on absorbance at 260 nm, which is in the UV range and not visible to the human eye (Figure 1). Tecan's multimode readers provide cuvette ports for DNA and RNA measurement (quartz or UV-transparent cuvettes must be used). Alternatively, Tecan's patented NanoQuant Plate™ (Figure 2) is ideal for smaller volumes (2 µl), higher throughput (16 samples at once), and more economical DNA / RNA quantification. 1 OD_{260} corresponds to different concentrations, depending on the type of nucleic acid being quantified (Table 1).

The ratio of absorption at 260 nm vs 280 nm is commonly used to assess DNA contamination of protein solutions, since proteins – in particular, the aromatic amino acids – absorb light at 280 nm[3,4]. It is generally acknowledged that pure DNA has a ratio of 2, and RNA, 1.8[5]. Recently researchers found that the A_{260}/A_{230} ratio also provides valuable information about the nucleic acid purity[6].

Typical detection limits for absorbance based DNA quantification are within the single digit ng/µl range.

Alternative.

LifeTechnologies' PicoGreen® and RiboGreen® quantification assays provide a broader dynamic range.

Sample protocol for DNA measurements.

1. Blank the reader with the same buffer used to dilute the DNA
2. Take an appropriate volume of sample (NanoQuant Plate: 2µl, cuvettes: volume depends on the min. / max. filling volume)
3. Measure OD_{260} and OD_{280}
4. If the OD_{260} value is greater than two, dilute samples
5. To calculate the concentration, multiply the OD_{260} by the concentration factor shown in Table 2 and your dilution factor, if applicable
6. Determine the purity by dividing the value for OD_{260} by the value for OD_{280}

[3] a b c d e Sambrook and Russell (2001). Molecular Cloning: A Laboratory Manual (3rd ed.). Cold Spring Harbor Laboratory Press. ISBN 978-0-87969-577-4.
[4] (Sambrook and Russell cites the original paper: Warburg, O. and Christian W. (1942). "Isolierung und Kristallisation des Gärungsferments Enolase". Biochem. Z. 310: 384–421.)
[5] Glasel, J.A. (1995) Validity of Nucleic Acid Purities Monitored by A260/A280 Absorbance Ratios, Biotechniques 18:62-63
[6] https://en.wikipedia.org/wiki/Nucleic_acid_quantitation

Instrument parameters.

			
Reader	**Infinite® 200 PRO**	**Spark**	**Spark Cyto**
Assay	**DNA concentration**	**DNA concentration**	**DNA concentration**
Measurement Mode	Absorbance	Absorbance	Absorbance
Wavelength	260 nm	260 nm	260 nm
Bandwidth	5 nm	default	default
Flashes	25	25	25
Settle time	0 ms	0 ms	0 ms

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

LABEL-FREE PROTEIN QUANTIFICATION IN LOW VOLUMES.

One-click application to quantify a know protein.

Figure 1: Snapshot of the SparkControl One-click application for the Protein Quantification App.

TECHNOLOGY.

The quantification of protein concentration in a biological sample is a very common application in biological laboratories. There are several possibilities to determine the concentration, including ultraviolet (UV) absorbance, reagent-based assays, and immunoassay technologies. The suitability of each assay depends on the sample type and the available volume. However, any dye-based assay might be more sensitive, especially in “protein mixtures,” but also requires more effort and costs in terms of assay preparation.

A quick and easy approach to determine the concentration of a specific protein in solution is the UV-based method. Amino acids with aromatic side chains – tryptophan, tyrosine, etc. – provide distinctive UV absorbance at 280 nm. The Spark and Spark Cyto, both equipped with high-speed absorbance monochromators, can be used to estimate the protein concentration in a sample by the linear correlation between the amount of protein in the sample and the optical density (OD) measured at 280 nm. For calculating the protein concentration in mg/ml, the Beer-Lambert law is followed. Besides the measured OD280 value, the extinction coefficient of the protein must be known, as well as the pathlength/distance of the light passing through the sample. In addition, the molecular weight of the protein in solution is required. A significant benefit is the combination of the method with Tecan’s low-volume NanoQuant Plate. This not only allows for label-free protein detection with minimal sample preparation but also enables downscaling the sample volume to only 2 µl, which saves valuable sample volume and therefore overall costs.


1715
1 2
A
B
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D
E
F
G
H

BCA, MODIFIED LOWRY AND BRADFORD ASSAYS – PROTEIN QUANTIFICATION.

Protein quantification assays with absorbance readout.

Figure 1: Bradford Protein Assay measured in cuvettes, showing increasing protein concentrations.

ASSAY OVERVIEW.

Technology.

Absorbance

Principle, Major application.

All three assays are designed to determine the protein concentration of a sample. For detection, a liquid reagent needs to be added to the samples. This reagent interacts with the proteins, leading to a visible color change (Figure 1) that is directly proportional to the concentration. Absolute concentrations are calculated using a standard curve.

Provider.

Various companies have established their own assays for this purpose. The main differences between the various assays are the dynamic range and the measurement wavelength.

Mechanism.

The **BCA**™ Protein Assay (Thermo Scientific Pierce) uses bicinchoninic acid (BCA) for colorimetric quantification of total protein in a sample[7]. The method is based on the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium to form a colored watersoluble chelate that can be measured at its absorption maximum of 562 nm. The linear working range for BSA is 20 to 2000 µg/ml[8].

The **Bradford** Protein Assay (BioRad) is based on the Coomassie® Brilliant Blue G-250 dye which binds to basic and aromatic amino acid residues, particularly arginine. This induces a shift of the dye's absorbance maximum from 465 nm to 595 nm. The Bradford assay can be performed as a microassay procedure, with a linearity range of 125 to 1,000 µg/ml BSA[9].

In the **Modified Lowry** Protein Assay (Thermo Scientific Pierce), the protein reacts with cupric sulfate and tartrate in an alkaline solution, which results in formation of a tetradentate copper-protein complex, reducing the Folin-Ciocalteu Reagent. The absorbance of the blue, water-soluble product can be measured at 750 nm. The assay – tested with BSA protein[10] – exhibits good linearity in the range of 1 to 1500 µg/ml.

Alternatives.

Potential alternatives for protein quantification reach from absorbance-based methods using the protein extinction coefficient[11] to fluorescence based assays like NanoOrange from Thermo Fisher to even dedicated[12] devices.

[7] Smith, P.K., et al.: Measurement of protein using bicinchoic acid. Anal Biochem., 150, 76-85, 1985
[8] https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-assays-analysis/protein-assays.html
[9] http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4110065A.pdf
[10] https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-assays-analysis/protein-assays.html
[11] http://web.expasy.org/protparam/protparam-doc.html
[12] http://www.millipore.com/techpublications/tech1/an2222en

Instrument parameters.

Reader	Infinite 200 PRO	Spark	Spark Cyto
Assay	**BCA Assay**	**BCA Assay**	**BCA Assay**
Measurement Mode	Absorbance	Absorbance	Absorbance
Wavelength	565 nm	562 nm	562 nm
Bandwidth	9 nm	default	default
Flashes	25	25	25
Settle time	0 ms	0 ms	0 ms

Assay	Modified Lowry assay	Modified Lowry assay	Modified Lowry assay
Measurement Mode	Absorbance	Absorbance	Absorbance
Wavelength	750 nm	750 nm	750 nm
Bandwidth	9 nm	default	default
Flashes	25	25	25
Settle time	0 ms	0 ms	0 ms

Assay	Bradford assay	Bradford assay	Bradford assay
Measurement Mode	Absorbance	Absorbance	Absorbance
Wavelength	595 nm	595 nm	595 nm
Bandwidth	9 nm	default	default
Flashes	25	25	25
Settle time	0 ms	0 ms	0 ms

SUPPORT.

Links.

• http://lifesciences.tecan.com/multimode-plate-reader?p=Technology

• http://lifesciences.tecan.com/infinite200pro_readmodes

MTT[13]/MTS[14] ASSAY.

Cell viability / cytotoxicity assays with absorbance readout.

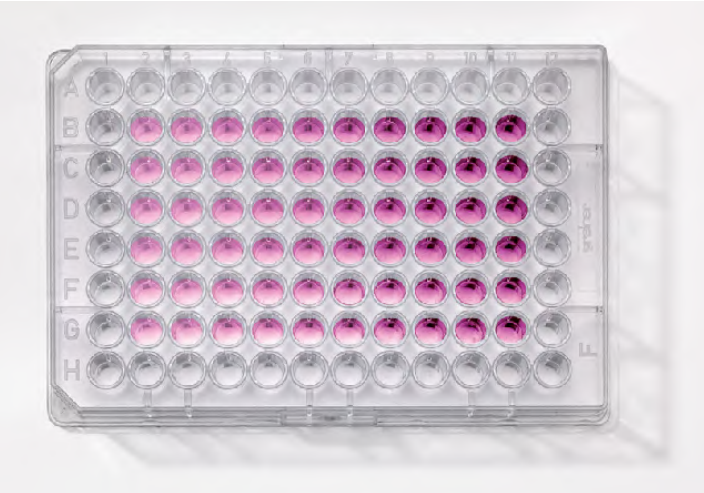

Figure 1: MTT assay in a 96-well format

ASSAY OVERVIEW.

Technology.

Absorbance

Principle, Major application.

The MTT and MTS assays are colorimetric (Figure 1) – and hence absorbance – assays that measure cell viability. MTT / MTS is taken up by the cells and processed to varying extents depending on the cell viability. Viable cells reduce more MTS / MTT to formazan, yielding a more intense purple color.

Mechanism.

While MTT assays need to use a reagent to make the formazan generated soluble, MTS assays yield water-soluble products, potentially making them homogeneous assays.

MTT assays use a solubilization reagent, such as dimethyl sulfoxide (DMSO) or isopropanol, to dissolve the non-water-soluble formazan product, yielding a colored solution that can be quantified by absorbance measurement at approximately 565 nm, dependent on the solvent employed[15].

MTS assays are an improved version of the MTT assay. Its reagents are reduced more efficiently within the cell than MTT, and the resulting product is water-soluble and less cytotoxic than the insoluble formazan used in the MTT assay. This makes it a one-step (homogeneous) assay, with the convenience of adding the reagent directly to the cell culture without the intermittent steps required in the MTT assay.

However, when MTS is used in a homogeneous way the assay becomes susceptible to colorimetric interference, as traces of colored compounds may remain in the microplate[16].

Provider.

MTT, MTS and similar reagents are available from various chemistry distributors, or as the CellTiter 96® AQueous One Solution Cell Proliferation Assay[17].

Alternatives.

An alternative to absorbance-based cell viability assays, are fluorescence-based systems using the redox indicator Resazurin to detect the cell's metabolic activity. These assays are characterized by a more convenient assay handling and higher sensitivity levels.

[13] MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-phenylditetrazolium bromide, a yellow tetrazole)

[14] MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

[15] Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods 65 (1-2): 55–63.

[16] Cory AH, Owen TC, Barltrop JA, Cory JG (1991). Use of an aqueous soluble tetrazolium/formazan assay for cellgrowth assays in culture. Cancer communications 3 (7): 207–12.

[17] https://at.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/celltiter-96-aqueous-one-solution-cell-proliferation-assay-_mts_/?catNum=G3582

Instrument parameters.

			
Reader	**Infinite 200 PRO**	**Spark**	**Spark Cyto**
Assay	**MTT assay/CellTiter Assay**	**MTT assay/CellTiter Assay**	**MTT assay/CellTiter Assay**
Measurement Mode	Absorbance	Absorbance	Absorbance
Wavelength	565 nm	565 nm	565 nm
Bandwidth	9 nm	3.5 nm	3.5 nm
Flashes	25	10	10
Settle time	0 ms	50 ms	50 ms

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

FLUORESCENCE INTENSITY – FI.

Light is absorbed and released (emitted).

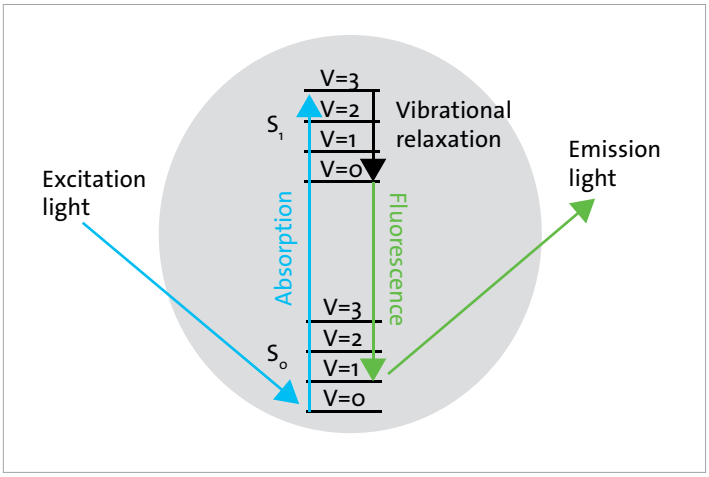

Figure 1: Jablonski diagram. S = electronic state, V = vibrational level. After photon absorption (= excitation), the molecule adopts a state of higher energy S_1 (= excited state) including several vibrationally excited substates. By vibrational relaxation, the molecule relaxes to the lowest excited S_1 state (black arrow). From this state the molecule relaxes into the vibrational states of S_0 by emitting light.

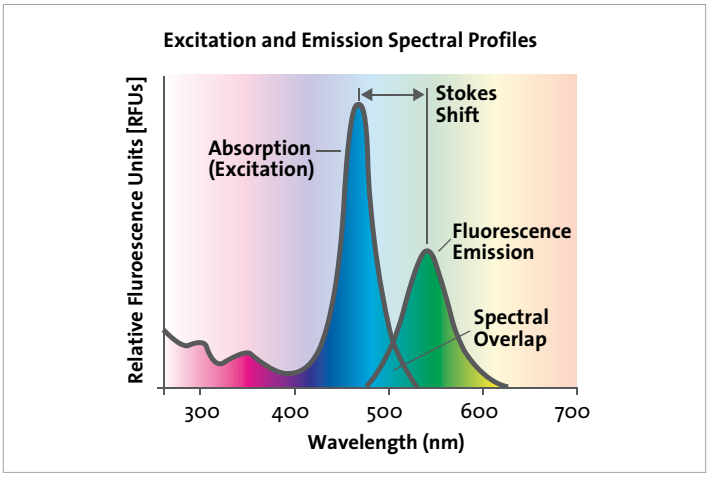

Figure 2: While the excitation spectrum describes how efficient it is to excite the fluorophore at a specific wavelength, the emission spectrum describes how efficient it is to detect the emitted light at any given wavelength. The Stokes shift describes the distance between the excitation and emission maximum, and is given in nanometers (nm).

TECHNOLOGY.

Fluorescence describes a molecule's ability to emit (release) previously absorbed light (Figure 1). The emission occurs almost instantly (within 1 ns = nano second) and, according to the laws of physics, the emitted light will always have a higher wavelength and hence a lower energy. A fluorescence spectrum consists out of an absorption (excitation) and emission spectrum (Figure 2).

Fluorescence labels (fluorophores) can be attached to any available biomolecule and used to answer quantitative, as well as qualitative, questions. For example, 'does the sample contain the fluorophore?' (qualitative), and 'how much of the fluorophore is in the sample?' (quantitative). Signals are quantified as Relative Fluorescence Units [RFU].

Major applications.

- PicoGreen® and RiboGreen® DNA / RNA quantification
- Resazurin-based cell viability assays
- Fluorescent proteins (GFP, RFP, YFP, etc.)
- ORAC (Oxygen Radical Absorbance Capacity) assay


Rinse
Prime
Rinse
Speed
Temperature
Off
Off

PICOGREEN® AND RIBOGREEN® DNA / RNA QUANTIFICATION.

High sensitivity, fluorescence-based DNA / RNA quantification.

Figure 1: DNA

ASSAY OVERVIEW.

Technology.

Fluorescence Intensity

Assay design and provider.

Thermo Fishers' PicoGreen[19] and RiboGreen[20] (Figure 1) quantification assays use a fluorescence approach to determine DNA and RNA concentrations. Using the Quant-iT PicoGreen dsDNA Assay Kit, you can selectively detect as little as 25 pg/ml of dsDNA in the presence of ssDNA, RNA, and free nucleotides. The assay is linear over three orders of magnitude, and has little sequence dependence, allowing you to accurately measure DNA from many sources.

RiboGreen RNA reagent is one of the most sensitive detection dyes for the quantification of RNA in solution, offering linear fluorescence detection in the range of 1 to 200 ng/ml of RNA.

Mechanism.

Both assays are easy to use; simply add the dye to the sample, wait five minutes, and detect the fluorescent signal.

Alternatives.

If sensitivity is not a major issue, it may be possible to perform DNA quantification using absorbance at 260 nm.

[19] https://www.thermofisher.com/order/catalog/product/P7589
[20] https://www.thermofisher.com/order/catalog/product/R11490

Instrument parameters.

			
Reader	**Infinite 200 PRO**	**Spark**	**Spark Cyto**
Assay	**PicoGreen/RiboGreen**	**PicoGreen/RiboGreen**	**PicoGreen/RiboGreen**
Measurement Mode	FI Top	FI Top	FI Top
Excitation wavelength Donor	485 (9) nm	485 (20) nm	485 (20) nm
Emission wavelength Donor	535 (20) nm	535 (25) nm	535 (25) nm
Lag time	0	0	0
Integration time	20 µs	default	default
Flashes	25	10	10
Mirror	automatic	automatic	automatic
Gain	optimal	optimal	optimal
Z-position	calculated from well	calculated from well	calculated from well
Settle time	0 ms	0 ms	0 ms

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

RESAZURIN ASSAY.

A Fluorescence Intensity-based cell proliferation assay.

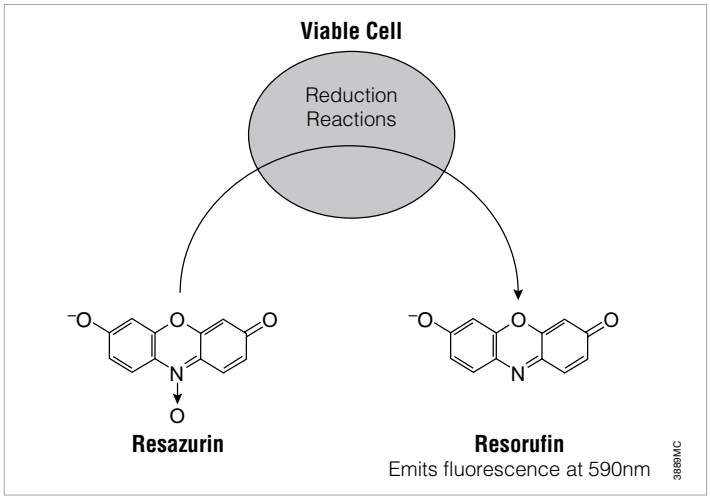

Figure 1: Viability dependet conversion of Resazurin to Resorufin (Promega)

ASSAY OVERVIEW.

Technology.

Fluorescence Intensity

Major application.

Resazurin is a chemical compound that is frequently used for cell viability assays.

Provider.

Resazurin was initially used for bacterial studies, but is now also available for eukaryotic cell-based applications under brand names such as the alamarBlue® assay[21] (Thermo Fisher) and CellTiter-Blue® Cell Viability Assay[22] (Promega).

Mechanism.

Resazurin is a redox indicator that can be added directly to cells. Viable cells convert the dark blue, oxidized form of the dye (resazurin) into a red, fluorescent reduced form called resorufin (Ex: 570 nm; Em: 590 nm). The amount of fluorescence or absorbance is proportional to the number of living cells, and corresponds to the cell's metabolic activity. Damaged and non-viable cells have lower innate metabolic activity, and therefore generate a proportionally lower signal than healthy cells. The system is specific for cell viability as non-viable cells rapidly lose metabolic capacity and do not reduce resazurin. Consequently, a fluorescent signal[23] is not generated.

Alternatives.

PrestoBlue Cell Viability Reagent[24], a new development from Thermo Fisher that offers much shorter incubation times.

[21] https://www.thermofisher.com/ch/en/home/life-science/cell-analysis/fluorescence-microplate-assays/microplate-assays-cell-viability/alamarblue-assay-cell-viability.html?ef_id=31d652600ec71bc783d43536f977baee:G:s&s_kwcid=AL!3652!10!77378255351814!77378305105749&cid=bid_pca_iva_r01_co_cp1359_pjt0000_bid00000_0se_bng_bt_pur_con&msclkid=31d652600ec71bc783d43536f977baee

[22] https://www.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/celltiter_blue-cell-viability-assay/?catNum=G8080

[23] O'Brien, J.; Wilson, I.; Orton, T.; Pognan, F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur. J. Biochem. 2000, 267,5421–5426.

[24] https://www.thermofisher.com/ch/en/home/life-science/cell-analysis/fluorescence-microplate-assays/microplate-assays-cell-viability/prestoblue-cell-viability-reagent.html?ef_id=c0223a0be12c19bb58335d5d16fe8d70:G:s&s_kwcid=AL!3652!10!76828499239519!76828696129478&cid=bid_pca_iva_r01_co_cp1359_pjt0000_bid00000_0se_bng_bt_pur_con&msclkid=c0223a0be12c19bb58335d5d16fe8d70

Instrument parameters.

			
Reader	**Infinite 200 PRO**	**Spark**	**Spark Cyto**
Assay	**alamarBlue, CellTiter-Blue**	**alamarBlue, CellTiter-Blue**	**alamarBlue, CellTiter-Blue**
Measurement Mode	FI Bottom	FI Bottom	FI Bottom
Excitation wavelength Donor	560 (9) nm	560 (20) nm	560 (20) nm
Emission wavelength Donor	600 (20) nm	600 (25) nm	600 (25) nm
Lag time	0	0	0
Integration time	20 µs	default	default
Flashes	25	10	10
Mirror	automatic	automatic	automatic
Gain	optimal	optimal	optimal
Z-position	calculated from well	calculated from well	calculated from well
Settle time	0 ms	0 ms	0 ms

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

GFP (GREEN FLUORESCENT PROTEIN).

Fluorescent protein frequently used as an expression / activation reporter.

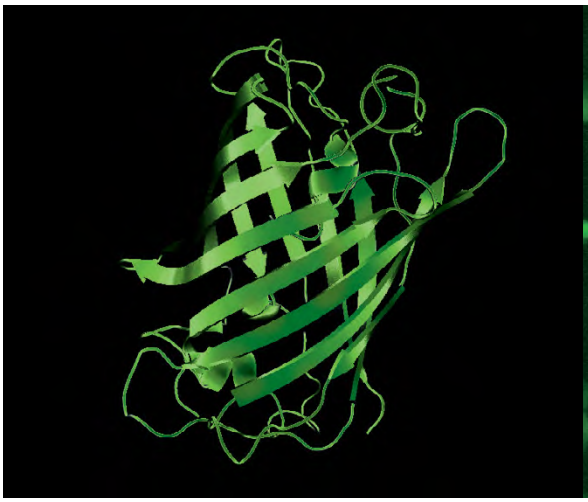

Figure 1: Protein structure of GFP

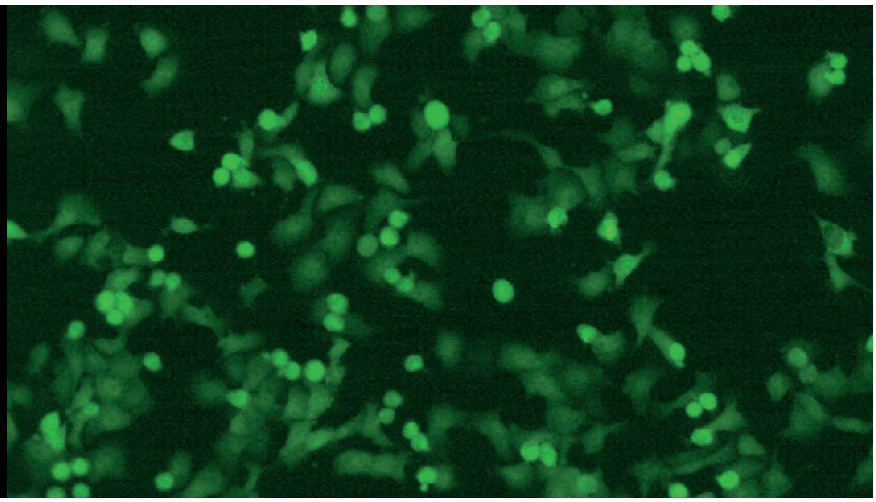

Figure 2: GFP-transfected eukaryotic cells

ASSAY OVERVIEW.

Technology.

Fluorescence Intensity

Principle.

GFP (Figure 1) is a protein derived from a jellyfish which has the ability to fluoresce in the green wavelength range and can be detected using standard FI measurements.

Major applications.

GFP can be used in an almost unlimited number of ways, for example as a BRET / FRET partner in binding studies, or for gene activation, where it is often fused / cloned to a gene of interest and co-expressed once the gene is activated (Figure 2). Commonly, it is used to differentiate between constitutive (permanent) and temporary expression. Constitutive expression is mostly used to monitor growth or proliferation of cells or bacteria, while temporary expression is used for gene activation studies.

Format, provider.

Due to multiple engineering efforts, an almost unlimited number of mutants exist, resulting in a large bandwidth of excitation and emission values. Some of these variants are commercially available, while others are published and therefore not protected. Consequently, only a selection of measurement parameters can be given, since the wavelength depends on the mutant type of the protein.

Alternatives.

Technology-wise, there are a lot of alternative fluorescent proteins available, such as CFP (cyan), YFP (yellow) and RFP (red). From an assay perspective, the alternative selected depends on the application. For gene expression studies, DLR® (Dual luciferase reporter assay) or GeneBLAzer® assays may be suitable. For FRET / BRET studies, fluorescent labels might be an alternative.

Instrument parameters.



Reader	**Infinite 200 PRO**	**Spark**	**Spark Cyto**
Assay	**GFP**	**GFP**	**GFP**
Measurement Mode	FI Bottom	FI Bottom	FI Bottom
Excitation wavelength Donor	485 (9) nm	485(20) nm	485(20) nm
Emission wavelength Donor	535 (20) nm	535(25)	535(25)
Lag time	0 µs	0 µs	0 µs
Integration time	20 µs	default	default
Flashes	25	15 (5x3, optimal read)	15 (5x3, optimal read)
Mirror	automatic	automatic	automatic
Gain	optimal	optimal	optimal
Z-position	calculated from well	calculated from well	calculated from well

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

TIME-RESOLVED FLUORESCENCE – TRF.

Light is absorbed and emitted for a relatively long period of time.

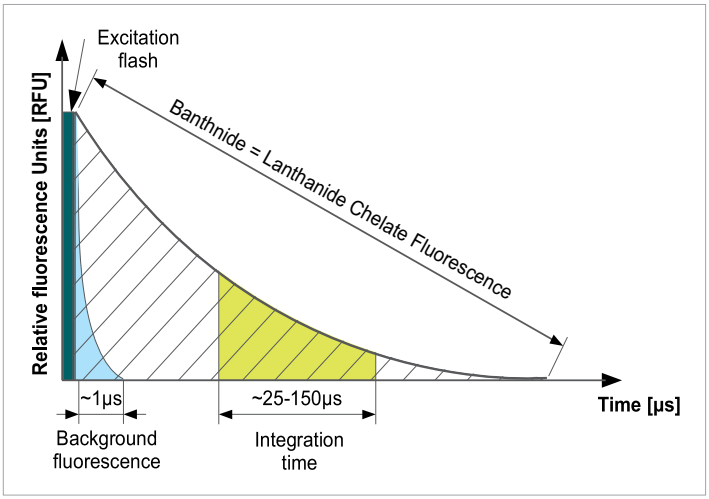

Figure 1: Schematic drawing of a time resolved emission spectrum

TECHNOLOGY.

TRF is similar to standard fluorescence, except that the light is emitted for a much longer period of time (Figure 1). The advantage of this is that the signal can be measured once all the background fluorescence (noise) has subsided, increasing the signal to noise ratio, and hence the sensitivity. Only lanthanides – also called rare earth metals – are capable of this kind of fluorescence[25].

In most cases, it is possible to substitute fluorescence applications with TRF to achieve higher sensitivity and / or lower background noise.

Major applications.

- DELFIA® – Dissociation-Enhanced Lanthanide Fluorescent Immunoassay

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

[25] Lakowicz, J. R. (1999). Principles of Fluorescence Spectroscopy. Kluwer Academic / Plenum Publishers


SPARK
CYTO
TECAN
cell imager

DELFIA® – DISSOCIATION-ENHANCED LANTHANIDE FLUORESCENT IMMUNOASSAY[26].

TRF-based alternative to absorbance-based ELISA.

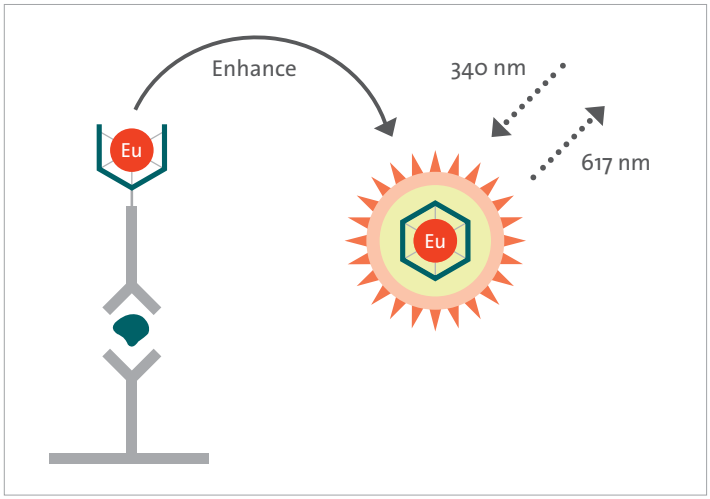

Figure 1: Schematic principle of DELFIA

ASSAY OVERVIEW.

Technology.

TRF – Time-Resolved Fluorescence

Principle, Provider.

Revvity offers the most common lanthanide chelates, including Europium (Eu), Samarium (Sm), Terbium (Tb) and Dysprosium (Dy), under the brand name DELFIA, making DELFIA a technology rather than a single assay.

Major applications.

In addition to the self-labeling kits, which allow users to label almost any biomolecule with the lanthanide chelates, Revvity offers pre-coupled antibodies and DNA probes. DELFIA is also available as a ready-to-go assay for cytotoxicity or cell proliferation studies. Other major applications include: receptor-ligand binding, enzyme assays, protein-protein and protein-DNA interaction studies.

Alternatives.

As a common application, it is used as an alternative approach to the well-established, absorbance-based ELISA[27].

Mechanism.

The biomolecule (antibody, DNA probe, etc.) used for detection is labeled with one of the lanthanide chelates. Assays are performed in an endpoint manner and only need to be read once, when all pipetting steps are complete. All steps are performed according to a standard ELISA protocol. Instead of a substrate an enhancement solution is added, that disconnects the chelate lanthanide-chelate complex from the antibody to increase the signal intensity.

[26] https://www.revvity.com/at-en/category/delfia-trf-assays
[27] Enzyme linked Immunosorbent assay

Instrument parameters.

			
Reader	**Infinite 200 PRO**	**Spark**	**Spark Cyto**
Assay	**Delfia**	**Delfia**	**Delfia**
Measurement Mode	FI Top (2 labels)	FI Top (2 labels)	FI Top (2 labels)
Excitation wavelength	340(35)	340(35)	340(35)
Emission wavelength	612(10)	612(10)	612(10)
Lag time	200 µs	100 µs	100 µs
Integration time	400 µs	400 µs	400 µs
Flashes	25	150	150
Mirror	automatic	automatic	automatic
Gain	optimal	optimal	optimal
Z-position	calculated from well	calculated from well	calculated from well
Settle time	0 ms	0 ms	0 ms

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

FLUORESCENCE RESONANCE ENERGY TRANSFER – FRET.

Light is absorbed, transferred to another fluorophore and then emitted.

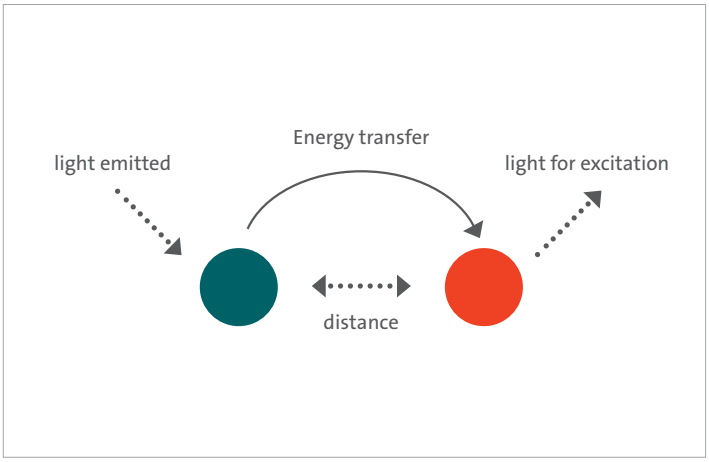

Figure 1: Schematic principle of FRET. Light emitted from the green molecule is used to excite the red molecule.

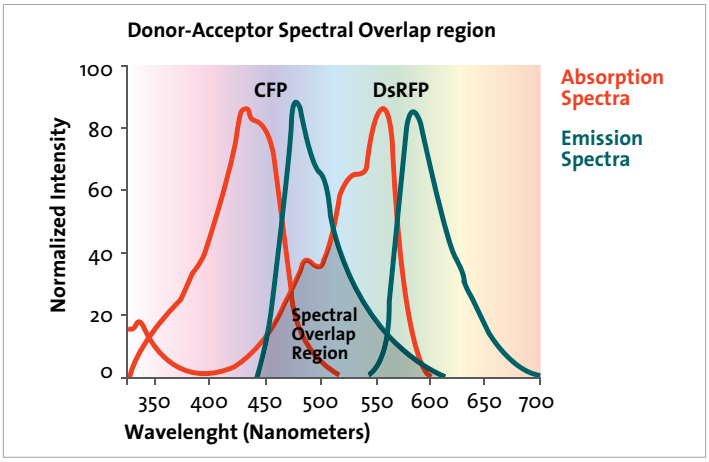

Figure 2: FRET is possible because the emission spectrum of CFP and the excitation spectrum of DsRFP overlap between 450 and 600 nm.

TECHNOLOGY.

As the name implies, FRET involves energy transfer between two fluorescent molecules (Figure 1). However, there are some specific requirements for this transfer to take place. Firstly, the emission spectrum of the donor fluorophore and the excitation spectrum of the acceptor fluorophore need to overlap (Figure 2), as the emission light of the donor fluorophore is used to excite the acceptor fluorophore. Secondly, the distance between the two fluorophores – the Förster radius – should be less than 10 nm (Figure 3)[28].

One way in which FRET is used is to determine if two biomolecules are in close proximity. In this case, both biomolecules must be labeled with fluorophores and then combined. After an incubation period, the assay is performed. Samples are excited at the donor excitation wavelength, and measured at the donor and acceptor emission wavelength. To compensate for well-to-well variation, for example from pipetting errors, the ratio of both values is calculated (ratiometric assay). If donor and acceptor are in close proximity, FRET will take place, otherwise only the emission signal of the donor is measurable.

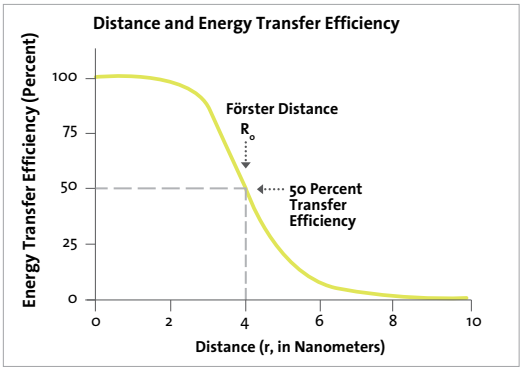

Figure 3: Förster radius – the distance where the FRET signal intensity is reduced to 50 %.

Major applications.

- GeneBLAzer and Tango™ GPCR Assay System

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

[28] Lakowicz, J. R. (1999). Principles of Fluorescence Spectroscopy. Kluwer Academic / Plenum Publishers

GENEBLAZER AND TANGO™ GPCR[29] ASSAY SYSTEM[30].

Gene activator assay with FRET readout.

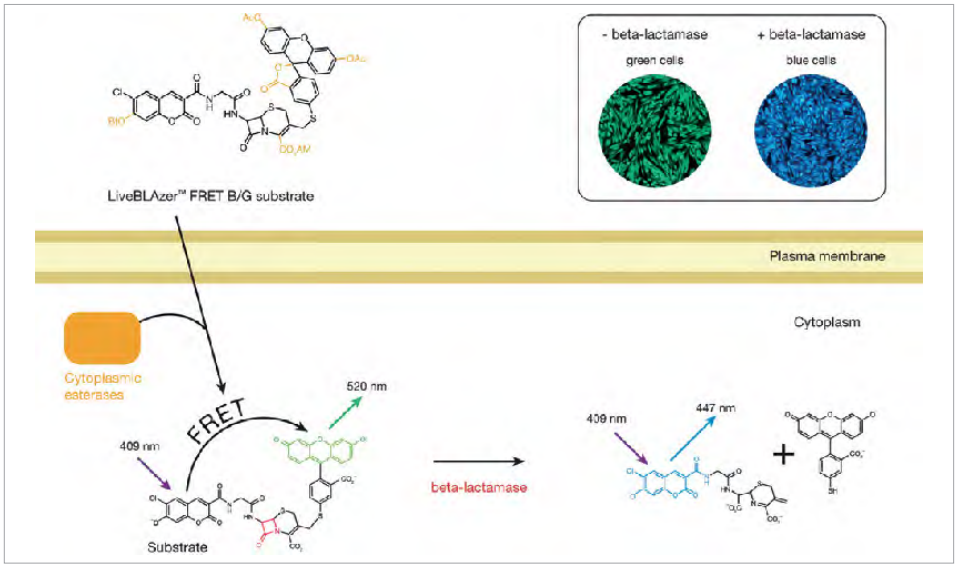

Figure 1: Principle of the GeneBLAzer assay. If the substrate is cleaved, the FRET signal is disrupted.

ASSAY OVERVIEW.

Technology.

Fluorescence Resonance Energy Transfer (FRET), ratiometric

Major application, Principle.

Thermo Fisher's GeneBLAzer assays are designed to monitor the activation of genes, including surface and intracellular reporters, a wide range of signal transduction pathways, ion channels and other transporters. The basis for the GeneBLAzer assay are cell lines possessing a β-lactamase[31] (BLA) gene under the control of a promotor which is downstream of the monitored target protein.

Provider.

The β-lactamase-transfected cell lines can either be purchased from Thermo Fisher or self-transfected. Tango cell lines are also based on the GeneBLAzer technology, but are designed exclusively for GPCR activation assays.

Mechanism.

As shown in Figure 1, after the transfection, cells are loaded with an engineered fluorescent substrate which is an assembly of two fluorophores: coumarin and fluorescein. If the target protein is inactive, BLA is not expressed and the substrate molecule remains intact. In this state, excitation of the coumarin results in FRET to the fluorescein moiety and emission of green light.

However, in the presence of BLA expression, the substrate is cleaved, causing the separation of the fluorophores, and FRET cannot occur. This results in the emission of a blue fluorescence signal from coumarin.

Reporter assays are often measured over several hours, or even days. During this time period the plate can either be shuttled between the incubator and the reader, or a temperature and gas controlled multimode reader such as the Infinite 200 PRO or Spark may be used.

Alternatives.

LiveBLAzer™, which is a combination of GeneBLAzer and resazurin.

[29] G-Protein Coupled Receptors: important group of cell surface receptors for cellular signaling
[30] https://www.thermofisher.com/ch/en/home/industrial/pharma-biopharma/drug-discovery-development/target-and-lead-identification-and-validation/g-protein-coupled/gpcr-cell-based-assays/geneblazer-theory.html
[31] β-lactamase is an enzyme that can cleave specific substrates

TIME-RESOLVED FLUORESCENCE RESONANCE ENERGY TRANSFER – TR-FRET.

FRET with a longer lifetime and hence a lower background.

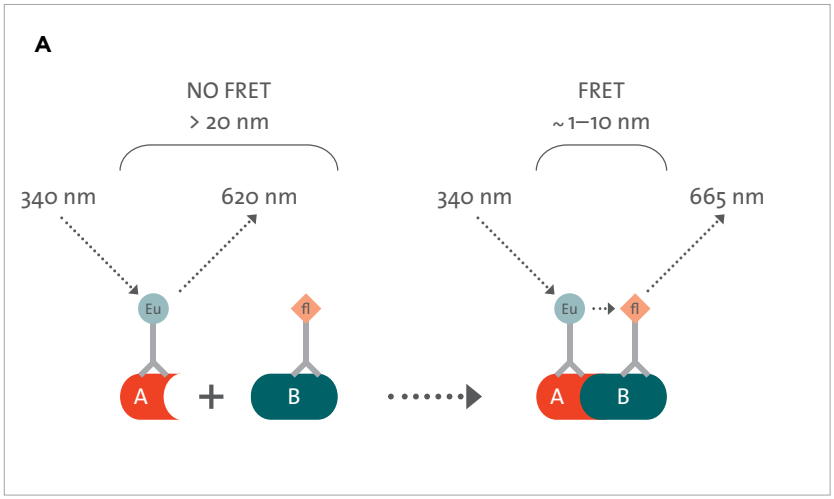

Figure 1: From a technology perspective TR-FRET is equivalent to FRET. In order to reduce the background of the assay, a lanthanide lable (e.g. Eu) and a standard fluorescence molecule (fl) as acceptor molecule.

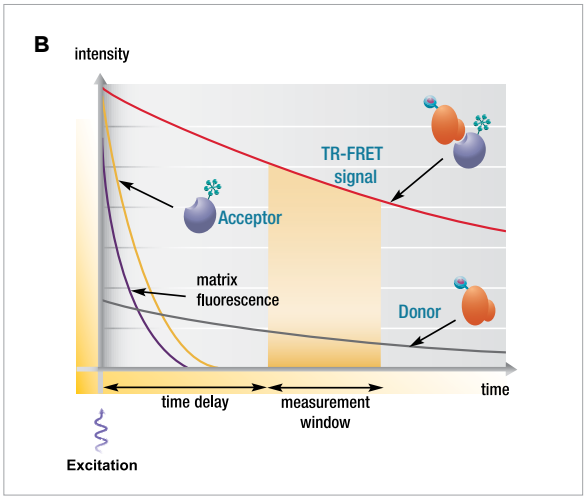

Figure 2: Signal vs. time plot showing the advantages of using lanthanide lables in a FRET process molecule.

TECHNOLOGY.

TR-FRET combines the advantages of Time-Resolved Fluorescence (TRF) with the functionality of Fluorescence Resonance Energy Transfer (FRET) (Figure 1). While most standard FRET assays have difficulties with high background resulting from the excitation of matrix molecules. This issue can be resolved by using time-resolved donor molecules. As shown in Figure 2, by the time donor and acceptor emission signals are measured all the unspecific background noise has vanished, giving a high signal to background noise ratio and therefore greater sensitivity.

Assay design

TR-FRET assays are commonly designed to detect whether molecules are in close proximity. This can be exploited to determine if, for example, a protein, peptide substrate, small molecule, phosphorylation, or acetylation is present, or if binding has occurred, for example a receptor-ligand interaction. A major limitation of this technology is that the maximum distance between the donor and the acceptor molecule cannot exceed 10 nm[32].

Major applications

- HTRF® – Homogeneous Time-Resolved Fluorescence
- Adapta® Universal Kinase Assay and Substrates
- LanthaScreen™ Kinase Activity Assays
- Transcreener® TR-FRET Assays

[32] Lakowicz, J. R. (1999). Principles of Fluorescence Spectroscopy. Kluwer Academic / Plenum Publishers

Instrument parameters.

Reader	**Infinite 200 PRO**
Assay	**GeneBLAzer**
Measurement Mode	FI Bottom (2 labels)
Excitation wavelength Donor	415(20)
Emission wavelength Donor	460(20)
Lag time	0 µs
Integration time	40 µs
Flashes	25
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms
Excitation wavelength Acceptor	415(20)
Emission wavelength Acceptor	535(25)
Lag time	0 µs
Integration time	40 µs
Flashes	25
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

HTRF® – HOMOGENEOUS TIME-RESOLVED FLUORESCENCE[33].

TR-FRET-based assay platform.

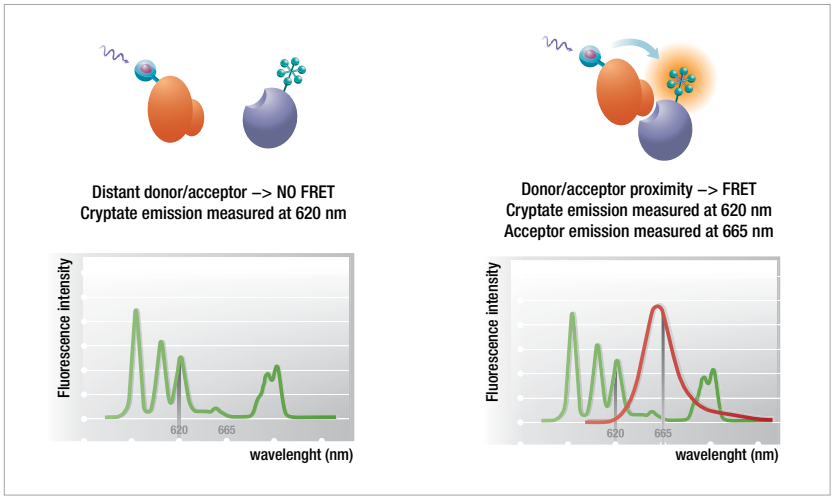

Figure 1: Mechanistic principle of the HTRF technology and fluorescence spectrum

ASSAY OVERVIEW.

Technology.

TR-FRET – Time-Resolved Fluorescence Resonance Energy Transfer

Provider.

HTRF is Revvity's TR-FRET-based assay platform, which provides a broad range of solutions.

Format, major applications.

Biomolecules for detection can either be self-labeled or purchased pre-labeled. Additionally, ready-to-go assays and pre-coupled antibodies are available for major targets, including GPCRs, with second messengers and binding assays, kinases, epigenetic enzymes, protein-protein interactions and biomarkers.

Mechanism.

HTRF is based on Eu^{3+} / Tb^{2+} cryptate donors and XL665 or d2 acceptors, which can be coupled to almost any biomolecule desired, including proteins, peptides, DNA and small molecules. The technology is based on no wash assay procedure detecting proximity events between donor and acceptor dyes.

The assay detection is obtained upon dispensing acceptor and donor conjugates to the sample to be assessed (e.g. enzymatic reaction mixture, cell lysate, or supernatant). No washing steps are required (homogeneous assay), and detection is performed after the completion of incubation, by measuring both specific donor and acceptor fluororescence (Figure 1). To compensate for well-to-well variation, the ratio of both values is calculated (hence ratiometric assay). Donor fluorescence will always be detected and used as an internal control, while an emission signal from the acceptor is only detected if both biomolecules are in close proximity and FRET occurs[33].

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

htrf
This reader is HTRF® compatible.

[33] https://www.revvity.com/at-en/content/htrf-principle-and-ease-use

Instrument parameters.

Reader	**Infinite 200 PRO**	**Spark**	**Spark Cyto**
Assay	**HTRF (Europium)**	**HTRF (Europium)**	**HTRF (Europium)**
Measurement Mode	FI Top (2 labels)	FI Top (2 labels)	FI Top (2 labels)
Excitation wavelength Donor	320(25)	320(25)	320(25)
Emission wavelength Donor	620(10)	620(10)	620(10)
Lag time	150 µs	100 µs	100 µs
Integration time	500 µs	400 µs	400 µs
Flashes	50	50	50
Mirror	automatic	automatic	automatic
Gain	optimal	optimal	optimal
Z-position	calculated from well	calculated from well	calculated from well
Settle time	0 ms	0 ms	0 ms
Excitation wavelength Acceptor	320(25)	320(25)	320(25)
Emission wavelength Acceptor	665 (8)	665(8)	665(8)
Lag time	150 µs	100 µs	100 µs
Integration time	500 µs	400 µs	400 µs
Flashes	50	50	50
Mirror	automatic	automatic	automatic
Gain	optimal	optimal	optimal
Z-position	same as Label 1	same as Label 1	same as Label 1
Settle time	0 ms	0 ms	0 ms

Reader	**Infinite 200 PRO**	**Spark**	**Spark Cyto**
Assay	**HTRF (Terbium)**	**HTRF (Terbium)**	**HTRF (Terbium)**
Measurement Mode	FI Top (2 labels)	FI Top (2 labels)	FI Top (2 labels)
Excitation wavelength Donor	340(35)	340(35)	340(35)
Emission wavelength Donor	620(10)	620(10)	620(10)
Lag time	150 µs	100 µs	100 µs
Integration time	500 µs	300 µs	300 µs
Flashes	50	50	50
Mirror	automatic	automatic	automatic
Gain	optimal	optimal	optimal
Z-position	calculated from well	calculated from well	calculated from well
Settle time	0 ms	0 ms	0 ms
Excitation wavelength Acceptor	340(35)	340(35)	340(35)
Emission wavelength Acceptor	665 (8)	665 (8)	665 (8)
Lag time	150 µs	100 µs	100 µs
Integration time	500 µs	400 µs	400 µs
Flashes	50	50	50
Mirror	automatic	automatic	automatic
Gain	optimal	optimal	optimal
Z-position	same as Label 1	same as Label 1	same as Label 1
Settle time	0 ms	0 ms	0 ms

ADAPTA® UNIVERSAL KINASE ASSAY AND SUBSTRATES.

Thermo Fisher's version of the ADP detection assay.

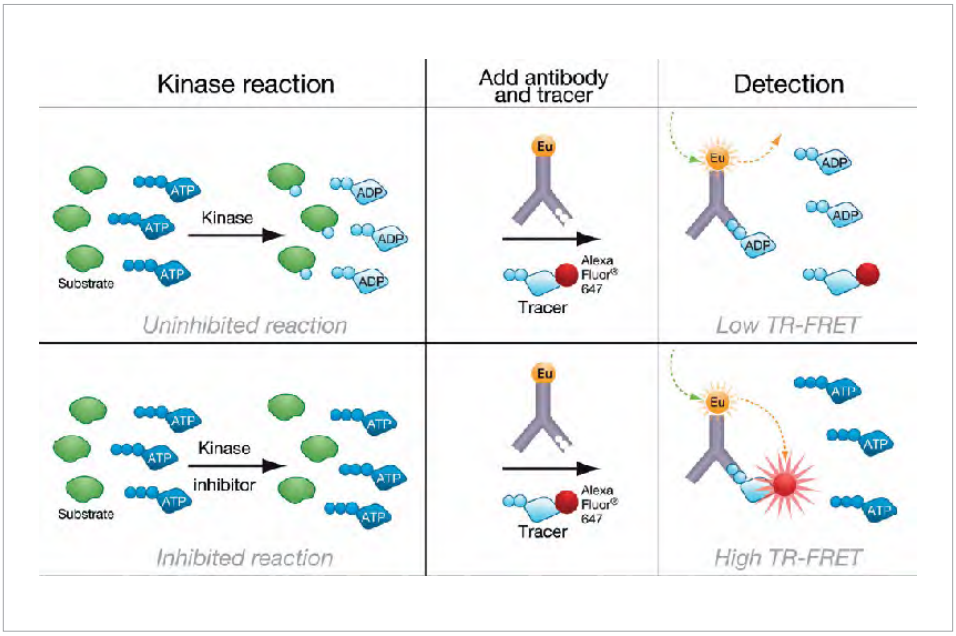

Figure 1: Schematic principle of the Adapta assay

ASSAY OVERVIEW.

Technology.

Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET), ratiometric

Principle, provider, major application.

Thermo Fisher's Adapta Universal Kinase Assay Kit is a homogeneous, fluorescence-based immunoassay for measuring the activity of ADP-producing enzymes, mainly kinases. Additionally, the Adapta assay is available for a selection of lipid- and peptide-based substrates. Thermo Fisher supplies europium-coupled antibody specific for ADP. In contrast to the Transcreener® platform, Adapta is only available for ADP.

Mechanism.

The ADP-specific antibody and the tracer are added to the sample. In an inhibited reaction (Figure 1), the monitored kinase produces no ADP and only the added, tracer-bound ADP molecule binds to the antibody, causing a high FRET signal.

Active kinases convert ATP to ADP. The free ADP competes with the tracer-bound ADP to bind to the antibody, resulting in a low FRET signal. Hence, the signal intensity is indirectly proportional to the activity of the kinase.

Instrument parameters.

Reader	**Infinite 200 PRO**
Assay	**Adapta Assay**
Measurement Mode	FI Top (2 labels)
Excitation wavelength Donor	340(35)
Emission wavelength Donor	620(10)
Lag time	100 µs
Integration time	200 µs
Flashes	10
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms
Excitation wavelength Acceptor	340(35)
Emission wavelength Acceptor	665(8)
Lag time	100 µs
Integration time	200 µs
Flashes	10
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms

SUPPORT.

Links.

• http://lifesciences.tecan.com/multimode-plate-reader?p=Technology

• http://lifesciences.tecan.com/infinite200pro_readmodes

LANTHASCREEN™ KINASE ACTIVITY ASSAYS[34].

Kinase activity assay with TR-FRET readout.

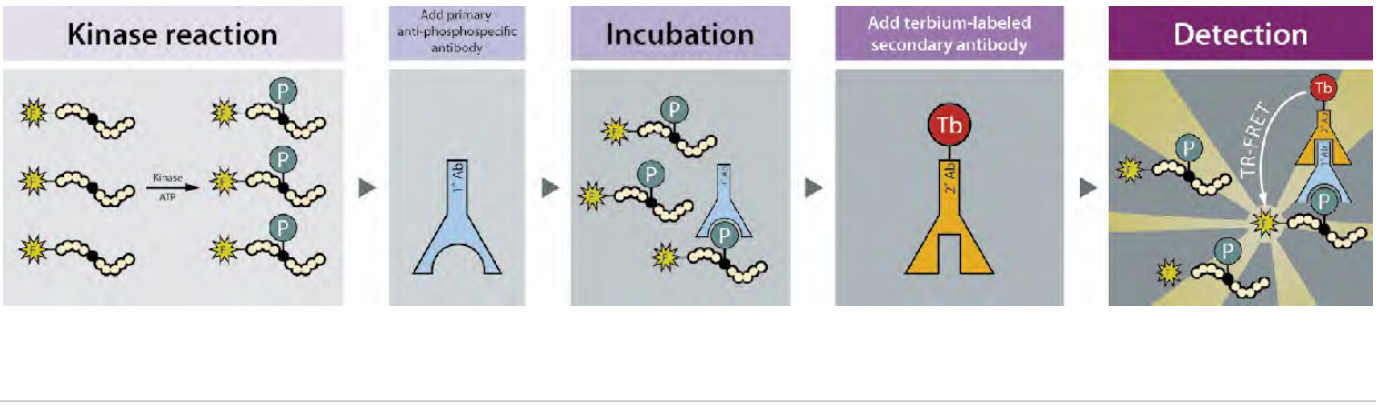

Figure 1: Schematic principle of the LanthaScreen assay

ASSAY OVERVIEW.

Technology.

Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET), ratiometric

Principle, provider, major applications.

LanthaScreen is a kinase activity assay sold by Thermo Fisher. Kinases are important cellular enzymes, and their major function is to add phosphate groups to peptide substrates. For researchers, it is important to know how active kinases are in the presence of certain inhibitors. LanthaScreen quantifies kinase activity by measuring the amount of phosphorylated substrate.

Format.

Thermo Fisher supplies a broad panel of fluorescein-labeled substrates and the corresponding lanthanide-labeled antibody specifically for the detection of phosphorylated substrates.

Mechanism.

Kinase and fluorescein-labeled substrates are incubated to enable phosphorylation. After incubation, a terbium-labeled antibody is added to the reaction (Figure 1).

Scenario 1 – kinase is active.

The substrate was phosphorylated, allowing the phospho-specific antibody to bind. The fluorescein and terbium labels are now in close proximity, resulting in a high FRET signal.

Scenario 2 – kinase is inactive.

No phosphorylation occurred, and therefore the antibody could not bind to the substrate. FRET cannot occur. The final result is a dimensionless number that is calculated as the ratio of the acceptor (fluorescein) signal to the donor (terbium) signal.

[34] https://www.thermofisher.com/at/en/home/industrial/pharma-biopharma/drug-discovery-development/target-and-lead-identification-and-validation/kinasebiology/kinase-activity-assays/lanthascreen-tr-fret-toolbox.html

Instrument parameters.

Reader	**Infinite 200 PRO**	**Spark**	**Spark Cyto**
Assay	**LanthaScreen (Terbium)**	**LanthaScreen (Europium)**	**LanthaScreen (Europium)**
Measurement Mode	FI Top (2 labels)	FI Top (2 labels)	FI Top (2 labels)
Excitation wavelength Donor	340(35)	340(35)	340(35)
Emission wavelength Donor	495(10)	620(10)	620(10)
Lag time	100 µs	100 µs	100 µs
Integration time	200 µs	400 µs	400 µs
Flashes	10	30	30
Mirror	automatic	automatic	automatic
Gain	optimal	optimal	optimal
Z-position	calculated from well	calculated from well	calculated from well
Settle time	0 ms	0 ms	0 ms
Excitation wavelength Acceptor	340(35)	340(35)	340(35)
Emission wavelength Acceptor	520(10)	665(8)	665(8)
Lag time	100 µs	100 µs	100 µs
Integration time	200 µs	400 µs	400 µs
Flashes	10	30	30
Mirror	automatic	automatic	automatic
Gain	optimal	optimal	optimal
Z-position	calculated from well	calculated from well	calculated from well
Settle time	0 ms	0 ms	0 ms

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

FLUORESCENCE POLARIZATION – FP.

Binding assay for biomolecules.

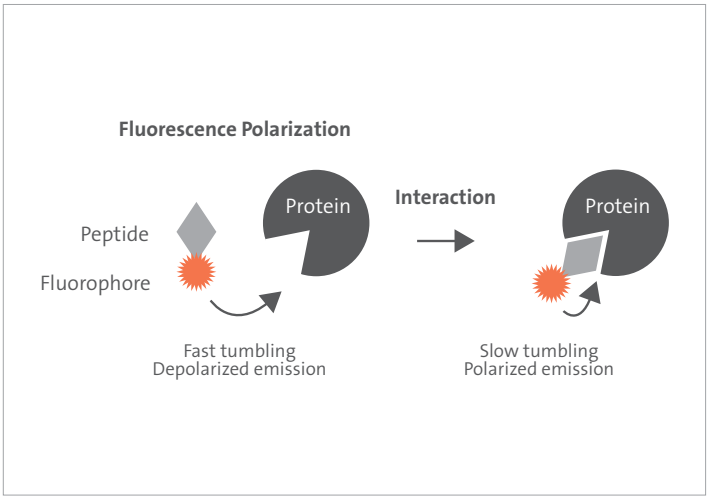

Figure 1: Schematic reprenstation of Fluorescence Polarization

$$mP = 1000 \times \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

Equation 1: Calculation of the polarization value. $I_{\parallel}$ = light parallel to the polarization plane. $I_{\perp}$ = light perpendicular to the polarization plane

TECHNOLOGY.

Fluorescence anisotropy is colloquially referred to as Fluorescence Polarization. On excitation with polarized light, the emission from many samples is also polarized. Rotational movements of the excited molecule destroy this correlation. The extent of polarization remaining depends on the size of the molecules measured: the bigger the molecules, the slower they rotate and the higher the conservation of the original polarization. Other influences include solvent viscosity, temperature and the lifetime of the excited state.[35]

The following metaphor is an easy way to explain FP. Imagine a little child playing in a field. While it is free, it can twist and turn as much as it wants in any direction. Once it is 'attached' to its mother's hand, the movements will slow down and get direction. This comparison can be used to show how FP detects molecular interactions (Figure 1). The little child represents the smaller of the interaction partners, and the mother the larger one. A fluorescent probe is attached to the small molecule to observe the turning and twisting movements. As long as there is no interaction between the small and the large molecule, the rotation of the fluorophore is fast and the emitted light depolarized. Once it binds to a larger interaction partner, its movements will slow down and the emitted light will preserve more and more of the original polarization.

Assay design.

A major application of FP is the detection of molecular interactions. FP assays require interaction partners to be different sizes, and the smaller molecule to be labeled with a fluorophore. Commercial assays commonly provide these labeled partners or substrates. The final result is a ratio of the polarization values, measured before and after addition of the suspected interaction partner. The polarization is calculated using the equation given below, measuring the intensity of emitted light in perpendicular and parallel planes.

Changes in polarization give information about the creation of interactions and their strength. A higher mP (milli-polarization) value represents a stronger interaction between the two molecules.

Major applications.

- PolarScreen™ Assays
- Transcreener® Assays
- Predictor™ hERG assay

[35] Lakowicz, J. R. (1999). Principles of Fluorescence Spectroscopy. Kluwer Academic / Plenum Publishers


te-inject.
INFINITE LUMI.
TECAN.

POLARSCREEN™.

FP-based kinase activity assay; FP-equivalent to LanthaScreen.

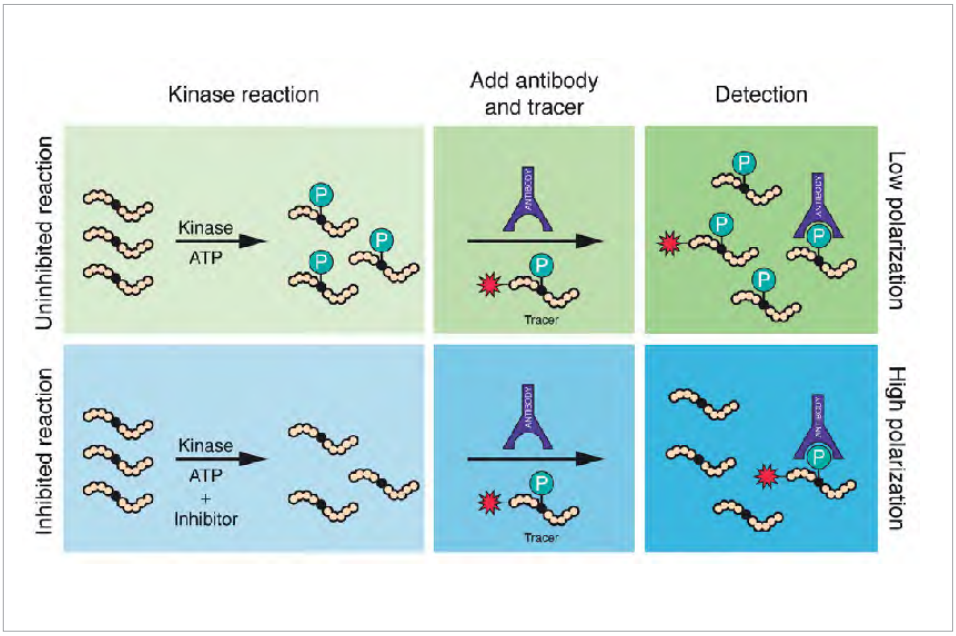

Figure 1: Schematic principle of the PolarScreen assay.

ASSAY OVERVIEW.

Technology.

Fluorescence Polarization (FP)

Provider, major application.

PolarScreen[36] is Thermo Fisher's version of a FP-based kinase activity assay. Thermo Fisher offers a panel of phospho-specific antibodies which detect peptide substrates when phosphorylated by protein kinases.

Mechanism.

The target kinase is incubated with a dedicated, unlabeled substrate (Figure 1). Antibody – specific for the phosphorylated- phosphorylation site of the substrate – and additional, tracer-bound substrate are added to the sample. If the unlabeled substrate's phosphorylation site remains unphosphorylated, for example due to an inactive enzyme, the antibody will only bind to the added, tracer-bound substrate, causing a low FP signal.

Active kinases will phosphorylate the non-tracer bound substrate, which then competes with the tracer-bound substrate for binding to the antibody, resulting in a high FP signal. Hence, the FP signal is directly proportional to the amount of phosphorylated substrate.

Format.

Fluorescence labels are available for green, red or far-red detection. Red fluorescence readouts help to reduce autofluorescence.

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

[36] https://www.thermofisher.com/us/en/home/industrial/pharma-biopharma/drug-discovery-development/target-and-lead-identification-and-validation/nuclear-receptor-biology/nuclear-receptor-biochemical-assays/polarscreen-competition-assays.html

Instrument parameters.

PolarScreen Green	
Reader	**Infinite 200 PRO**
Assay	**PolarScreen**
Measurement Mode	FP
Excitation wavelength Donor	485(20)
Emission wavelength Donor	535(25)
Lag time	0
Integration time	20 µs
Flashes	10
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms

PolarScreen Red	
Assay	**PolarScreen**
Measurement Mode	FP
Excitation wavelength Donor	535(25)
Emission wavelength Donor	590(20)
Lag time	0
Integration time	20 µs
Flashes	10
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms

PolarScreen Far Red	
Assay	**PolarScreen**
Measurement Mode	FP
Excitation wavelength Donor	610(20)
Emission wavelength Donor	670(40)
Lag time	0
Integration time	default
Flashes	10
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms

TRANSCREENER®.

Nucleotide (ADP, GDP, etc.) detection assay.

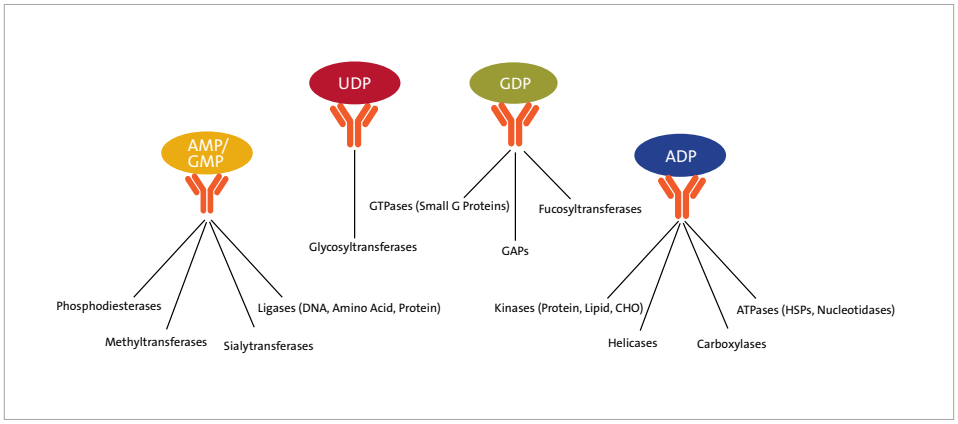

Figure 1: Transcreener targets

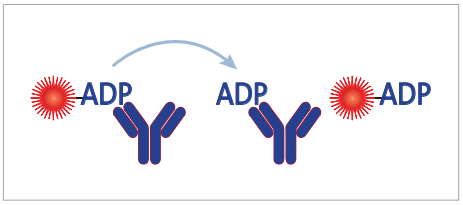

Figure 2: Transcreener principle

Assay	Readout
Transcreener ADP² Assays	FP, FI, TR-FRET
Transcreener AMP/GMP Assay	FP, FI
Transcreener GDP Assays	FP
Transcreener UDP Assays	FP

Table 1: Alternative readouts

ASSAY OVERVIEW.

Technology.

Fluorescence polarization (FP)

Fluorescence intensity (FI)

Time-resolved fluorescence resonance energy transfer (TR-FRET)

Principle, provider.

Bellbrook's Transcreener assays[37] are designed to detect various mono- and dinucleotides using FP, TR-FRET or FI detection mode. Four assays (Table 1) cover thousands of target enzymes, including any kinase, ATPase or GTPase. Transcreener is a universal assay method that can be used across entire families of nucleotide-dependent enzymes. All assays are based on different antibodies that show a high affinity for one specific nucleotide (Figure 1).

Mechanism.

The mechanism is the same for all Transcreener assays. The antibody is preloaded with the corresponding nucleotide, which is conjugated to a tracer molecule. All assays use a far red tracer that minimizes compound interference. For example, in the ADP² FP assay the detection mixture comprises of Alexa 633 ADP and a highly selective ADP monoclonal antibody. The Transcreener ADP² FP assay measures the progress of any enzyme that produces ADP by displacing the tracer by ADP thereby causing a decrease in fluorescence polarization. (Figure 2)

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

[37] https://www.bellbrooklabs.com/technical-resources/transcreener-faq/

Instrument parameters.

Reader	**Infinite 200 PRO**	**Spark**	**Spark Cyto**
Assay	**Transcreener FP**	**Transcreener FP**	**Transcreener FP**
Measurement Mode	FP	FP	FP
Excitation wavelength	610(20)	620(10)	620(10)
Emission wavelength	670(25)	670(25)	670(25)
Integration time	default	default	default
Flashes	10	30	30
Mirror	automatic	automatic	automatic
Gain	optimal	optimal	optimal
Z-position	calculated from well	calculated from well	calculated from well
Settle time	50 ms	100 ms	100 ms
Transcreener FI			
Assay	**Transcreener FI**	**Transcreener FI**	**Transcreener FI**
Measurement Mode	FI Top	FI Top	FI Top
Excitation wavelength	580(20)	580(20)	580(20)
Emission wavelength	620(20)	620(20)	620(20)
Lag time	n.a.	n.a.	n.a.
Integration time	20 µs	default	default
Flashes	25–100	30–100	30–100
Mirror	automatic	automatic	automatic
Gain	optimal	optimal	optimal
Z-position	calculated from well	calculated from well	calculated from well
Settle time	50 ms	100 ms	100 ms
Transcreener TR-FRET			
Assay	**Transcreener TR-FRET**	**Transcreener TR-FRET**	**Transcreener TR-FRET**
Measurement Mode	FI Top (2 labels)	FI Top (2 labels)	FI Top (2 labels)
Excitation wavelength Donor	320(25)	320(25)	320(25)
Emission wavelength Donor	620(10)	620(10)	620(10)
Lag time	150 µs	150 µs	150 µs
Integration time	500 µs	500 µs	500 µs
Flashes	10	30	30
Mirror	automatic	automatic	automatic
Gain	optimal	optimal	optimal
Z-position	calculated from well	calculated from well	calculated from well
Settle time	0 ms	0 ms	0 ms
Excitation wavelength Acceptor	320(25)	320(25)	320(25)
Emission wavelength Acceptor	665(8)	665(8)	665(8)
Lag time	150 µs	150 µs	150 µs
Integration time	500 µs	500 µs	500 µs
Flashes	10	30	30
Mirror	automatic	automatic	automatic
Gain	optimal	optimal	optimal
Z-position	calculated from well	calculated from well	calculated from well
Settle time	0 ms	n.a.	n.a.

AMPLIFIED LUMINESCENT PROXIMITY HOMOGENEOUS ASSAY – ALPHA.

FRET equivalent with reduced distance limitations and amplification.

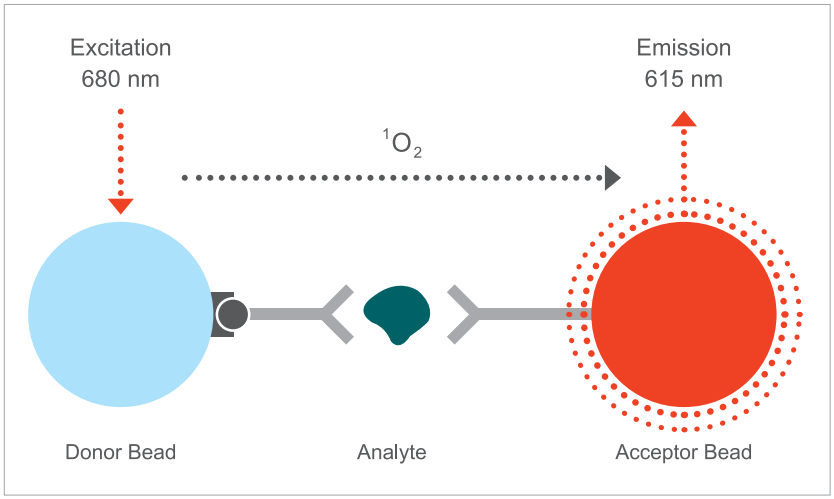

Figure 1: Schematic function of the Alpha technology.

TECHNOLOGY.

Mechanism.

Alpha[38] (Amplified Luminescent Proximity Homogeneous Assay) is a technology invented by Revvity's. The principle is similar to (TR-)FRET, since the Alpha Technology also relies on the interaction of an acceptor and a donor to yield a signal (Figure 1). However, instead of using simple fluorophores, chemically reactive beads are used, and the chemistry that produces the signal is also different. The main advantage of Alpha is that the distance between the interaction partners can be up to 200 nm, compared to 10 nm for (TR-)FRET. Furthermore, there is an amplification effect which increases the sensitivity of the assay. Background is reduced because the emission wavelength is lower than the excitation wavelength.

Assay design.

AlphaScreen is a bead-based screening technology developed by Revvity for fast and reliable detection of biological interactions. The AlphaScreen chemistry employs donor and acceptor beads that can be linked to various types of biologically relevant molecules. The phthalocyanine photosensitizer molecules contained in the AlphaScreen donor beads convert ambient oxygen into large quantities of singlet oxygen when excited by a high energy light source at a wavelength of 680 nm. The singlet oxygen molecules are able to cover a distance of up to 200 nm during their half-life of approximately 4 µs. If AlphaScreen acceptor beads are in close proximity to the donor beads, due to a biological binding event between their coupling partners, the singlet oxygen molecules are able to initiate a cascade of energy transfer steps in the acceptor beads, ultimately resulting in the generation of a strong light emission in the range of 520-620 nm. Due to the amplified signal generation, even small amounts of biological analytes can be detected.

AlphaLISA is a homogeneous, no-wash alternative to conventional ELISA assays based on Revvity's bead-based Alpha technology. AlphaLISAs can be set up as sandwich or competitive immunoassays to detect and quantify molecules of interest in biological samples. High energy excitation of photosensitizer molecules within the AlphaLISA donor beads at 680 nm converts ambient oxygen to singlet oxygen, which is able to react with the chemistry in the acceptor beads if these are in close proximity. A cascade of energy transfer steps ultimately results in the generation of a strong output signal at 615 nm, indicating specific binding between the molecules attached to the two bead types.

[38] https://resources.revvity.com/pdfs/009625_02%20GDE%20TSA%20IHC%20IC%20IF_V4.pdf

The fluorophores embedded in the AlphaLISA acceptor beads produce a narrower bandwidth signal than the acceptor beads used for classical AlphaScreen assays. This makes AlphaLISAs less prone to signal interference at wavelengths of <600 nm, increasing the sensitivity and robustness of the assay. The no-wash nature of this assay makes it easy to use, and the use of dedicated AlphaLISA optics permits the analysis of target molecules in blood and serum by drastically reducing the effect of hemoglobin within a sample.

AlphaPlex™ is a homogeneous, all-in-one-well multiplexing reagent technology that provides highly sensitive detection of a wide range of analytes in a simple no-wash assay format based on Revvity's proven Alpha Technology. AlphaPlex assays can quantify multiple analytes in a single well.

Alpha technologies' versatility offers the possibility to assay many biological targets, including enzymes, receptor-ligand interactions, low affinity interactions, second messenger levels, DNA, RNA, proteins, protein-protein interactions, peptides, sugars and small molecules[38].

Major applications.

- AlphaScreen
- AlphaLISA
- AlphaPlex

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

AlphaScreen / AlphaLISA.

Alpha Technology assay platforms.

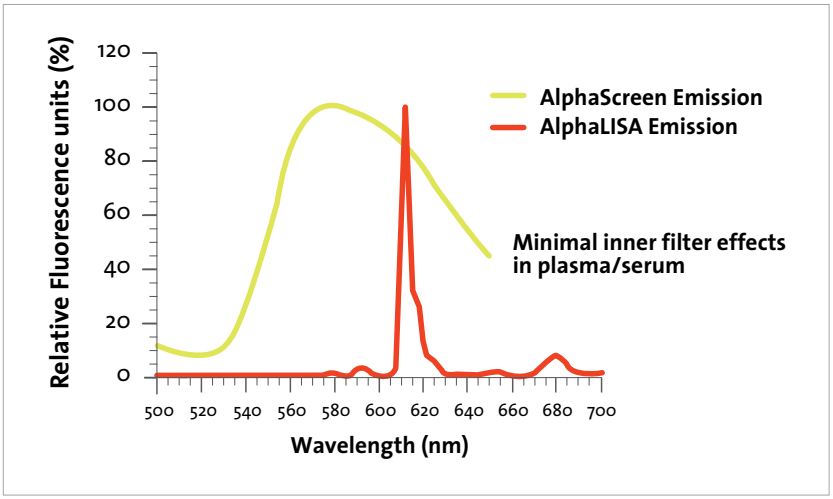

Figure 1: Emission spectra of TAR (AlphaScreen) and europium (AlphaLISA) acceptor beads.

ASSAY OVERVIEW.

Technology.

Alpha Technology

Provider, Format.

AlphaScreen and AlphaLISA are Revvity's Alpha Technology[39] assay / reagent platforms. Revvity supplies donor beads coupled to streptavidin, and blank acceptor beads for self-labeling. Additionally, acceptor beads can be purchased precoupled to antibodies specific for a broad range of targets.

Principle.

AlphaLISA is a development targeting laboratories working with crude blood samples, as the autofluorescence of hemoglobin overlaps with the emission peak (Figure 1) of the AlphaScreen acceptor. The main difference between AlphaLISA and AlphaScreen is that the AlphaLISA emission peak (europium emission) of the acceptor bead is smaller than that of the AlphaScreen assay (rubrene emission).

Mechanism.

For a closer description of the assay mechanism, please refer to the Alpha Technology section.

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

[39] https://resources.revvity.com/pdfs/009625_02%20GDE%20TSA%20IHC%20IC%20IF_V4.pdf

Instrument parameters.

Reader	**Spark**	**Spark Cyto**
Assay	**AlphaScreen**	**AlphaScreen**
Measurement Mode	Alpha Technology	Alpha Technology
Excitation wavelength	n.a.	n.a.
Emission wavelength	n.a.	n.a.
Excitation time	100 ms	100 ms
Integration time	300 ms	300 ms
Wavelength	520-620 nm	520-620 nm
Gain	n.a.	n.a.
Settle time	0 ms	0 ms
Temperature correction	activated	activated

Assay	**AlphaLISA**	**AlphaLISA**
Measurement Mode	Alpha Technology	Alpha Technology
Excitation wavelength	n.a.	n.a.
Emission wavelength	n.a.	n.a.
Excitation time	100 ms	100 ms
Integration time	300 ms	300 ms
Wavelength	610-635 nm	610-635 nm
Gain	n.a.	n.a.
Settle time	0 ms	0 ms
Temperature correction	activated	activated

Assay	**AlphaPlex**	**AlphaPlex**
Measurement Mode	Alpha Technology	Alpha Technology
Excitation wavelength	n.a.	n.a.
Emission wavelength	n.a.	n.a.
Excitation time	100 ms	100 ms
Integration time 1	300 ms	300 ms
Integration time 2	300 ms	300 ms
Wavelength 1	610-635 nm	610-635 nm
Wavelength 2	535-560 nm	535-560 nm
Gain	n.a.	n.a.
Settle time	0 ms	0 ms
Temperature correction	activated	activated

LUMINESCENCE – LUMI.

Light is emitted from the sample.

Figure 1: Firefly

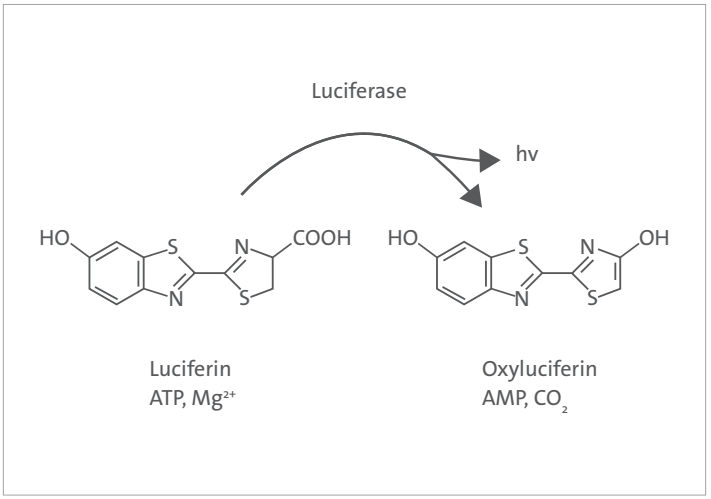

Figure 2: Substrate cleavage by luciferase

TECHNOLOGY.

Luminescence is widely known as a reaction that causes the release of light. It can be caused by chemical reactions, electrical energy, subatomic motion, or stress on a crystal. For molecular biology, bioluminescence is the most important of the various luminescence reactions. At the core of this technology is the luciferase enzyme. Luciferases (Firefly, Renilla) (Figure 1) convert a substrate into an excited state (Figure 2). When returning to the ground state, a photon (light) is released (emitted).

One striking difference between fluorescence and luminescence is that luminescence requires no excitation light. This reduces the background to almost zero, resulting in better sensitivity.

Various forms of luminescence can be differentiated.

1. Glow luminescence, which generates stable and measurable light up to several hours, for example the BioThema ATP assay
2. Flash luminescence, which is characterized by rapid, but short-lived, light generation, for example DLR and Aequorin (injectors are required for flash luminescence)
3. Multicolor luminescence, such as BRET$^{1/2/3/e}$ and Chroma-Glow™

Major applications.

- Dual-Luciferase® Reporter Assay (DLR™)
- Bio Thema ATP detection kit
- BRET (Bioluminescence Resonance Energy Transfer)
- NanoBRET™

ATP DETECTION (BIOTHEMA KIT[40]).

ATP detection kit based on luminescence.

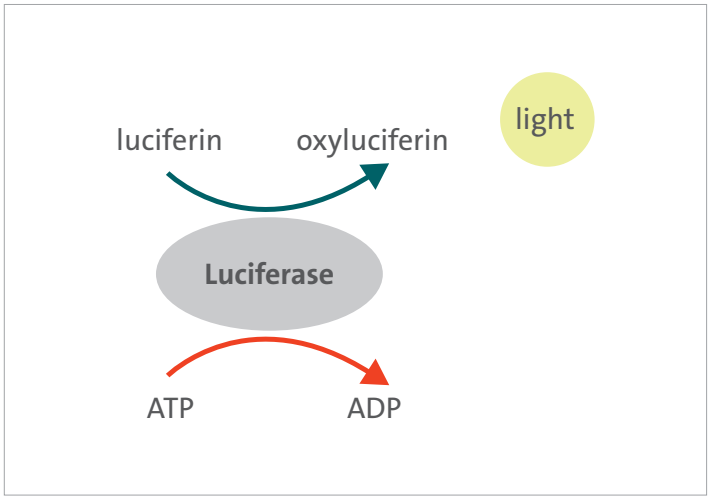

Figure 1: Schematic principle of luminescence caused by a luciferase based on the conversion of ATP.

ASSAY OVERVIEW.

Technology.

Luminescence (Glow)

Principle, provider.

ATP (Adenosine-Tri-Phosphate) is a molecule for short time energy storage and required for almost any reaction in living organisms. Biothema's ATP detection kit measure and quantifies the level of ATP and correlates it to the activity of an enzyme or cells.

Format.

The kit consists of a luciferase, the substrate and an ATP standard to quantify the signal of the sample.

Mechanism.

The luciferase and substrate are added to the sample. If ATP is present, the luciferase converts the substrate and light is released (Figure 1). If no ATP is present, no light is released. The more ATP is present in the sample the stronger is the signal. In a last step the signal is quantified by comparing it to a standard ATP curve.

Major applications.

Major applications include ATP detection, cell proliferation, cytotoxicity, enzymatic monitoring.

[40] http://biothema.se/products/kits /

Instrument parameters.

			
Reader	**Infinite 200 PRO**	**Spark**	**Spark Cyto**
Assay	**Biothema ATP**	**Biothema ATP**	**Biothema ATP**
Measurement Mode	Luminescence	Luminescence	Luminescence
Integration time	1000 ms	1000 ms	1000 ms
Attenuation	automatic	automatic	automatic
Settle time	0 ms	0 ms	0 ms

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

DUAL-LUCIFERASE® REPORTER ASSAY (DLR™)[41].

Luminescence-based normalizable gene activator assay.

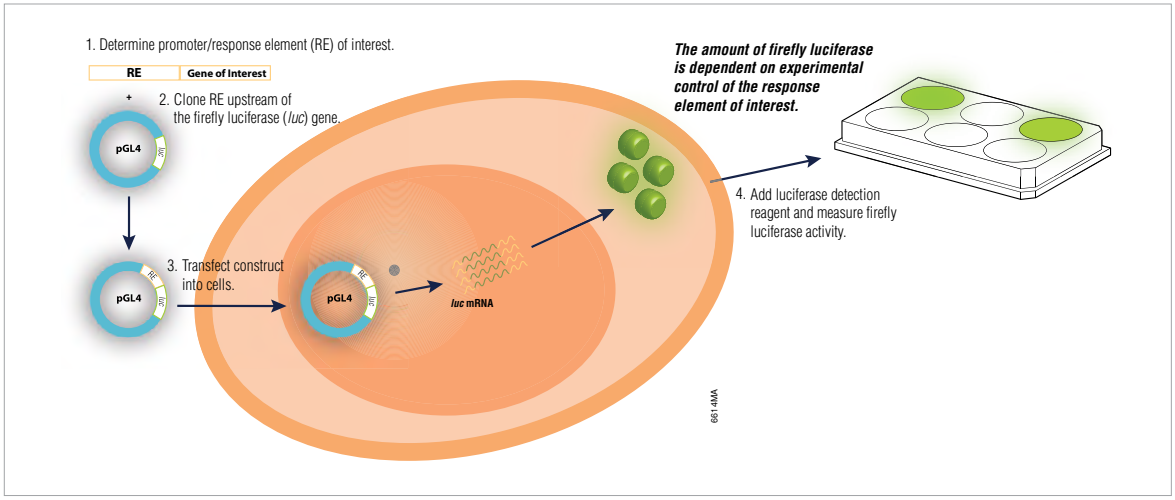

Figure 1: DLR assay mechanism

ASSAY OVERVIEW.

Technology.

Luminescence (flash)

Purpose, provider, major application.

Promega's DLR assay measures gene activation / expression using a luminescence-based readout. One particular challenge of gene activator assays is quantification of the result. The DLR assay resolves this issue by normalizing the output using two luciferases. While one luciferase measures the expression of the gene of interest, the second luciferase measures the expression of a housekeeping gene[42], which is used to normalize the signal. Promega has two series of firefly and Renilla luciferase vectors – pGL4 and pRL – designed for use with the DLR assay systems.

Mechanism.

Cells need to be transfected with both luciferase reporter genes (Figure 1). The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a 'glow-type' luminescent signal. After quantifying the firefly luminescence, the reaction is quenched, and the Renilla luciferase reaction simultaneously initiated, by adding Stop & Glo® Reagent to the tube. The Stop & Glo Reagent produces a 'glow-type' signal from the Renilla luciferase, which decays slowly over the course of the measurement.

DLReady™

[41] https://at.promega.com/products/luciferase-assays/reporter-assays/dual_luciferase-reporter-assay-system/?catNum=E1910
[42] Housekeeper genes are proteins or enzymes that are constitutionally expressed in most cells as for instance the DNA polymerase

Instrument parameters.

			
Reader	**Infinite 200 PRO**	**Spark**	**Spark Cyto**
Assay	**DLR**	**DLR**	**DLR**
Measurement Mode	Luminescence	Luminescence	Luminescence
	well-wise	well-wise	well-wise
Integration time	10,000 ms	10,000 ms	10,000 ms
Output	counts/sec	counts/sec	counts/sec
Attenuation	automatic	automatic	automatic
Settle time	0 ms	0 ms	0 ms
Injections	Injector A: 100 µl (refill for every injection), wait 3 s	Injector A: 100 µl (refill for every injection), wait 3 s	Injector A: 100 µl (refill for every injection), wait 3 s
	Injector B: 100 µl (refill for every injection), wait 3 s	Injector B: 100 µl (refill for every injection), wait 3 s	Injector B: 100 µl (refill for every injection), wait 3 s

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

BRET (BIOLUMINESCENCE RESONANCE ENERGY TRANSFER).

A FRET modification using the donor fluorophore as the light source.

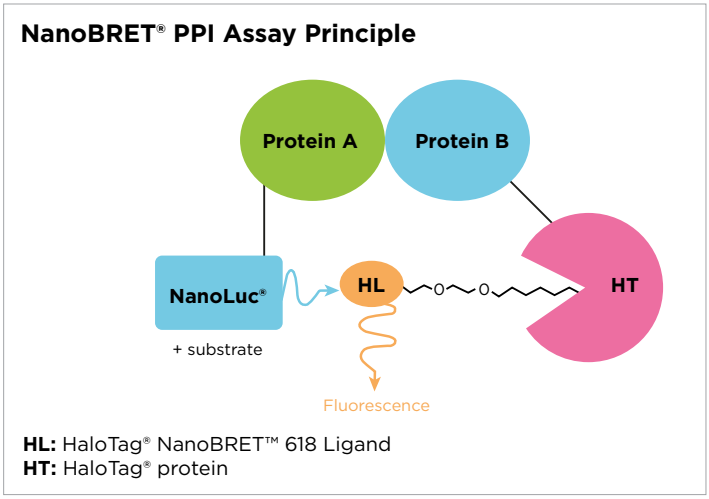

Figure 1: Principle of NanoBRET.

TECHNOLOGY.

BRET is a modification of FRET, the main difference being that the donor fluorophore is substituted by a luciferase. A major application for BRET is interaction studies. The advantage is that no excitation light is needed, and hence the background is much lower. BRET itself is a technology, and therefore can't be commercialized. However, various companies offer proprietary assay components for this technology, for example BRET$^{1/3/e}$ are an unlicensed technology, while BRET^{2} is a licensed product of Revvity.

The difference between BRET^{1} and BRET^{2} lies in the selection of the donor and acceptor proteins / fluorophores. This results in a shift of the excitation and emission wavelength.[43]

A new improved chemistry version of BRET, NanoBRET™, combines an extremely bright NanoLuc® luciferase with a means for tagging intracellular proteins with a long-wavelength fluorophore (HaloTag), providing a better dynamic range and sensitivity (Figure 1).[44]

[43] Bacart J, Corbel C, Jockers R, Bach S, Couturier C: The BRET technology and its application to screening assays. Biotechnol J 2008, 3:311-324.
[44] Machleit et al. NanoBRET–A novel BRET platform for the analysis of protein-protein interactions. ACS Chem. Biol., 2015, 10 (8), pp 1797–1804.

Compatible readers.

Reader	Infinite 200 PRO	Spark	Spark Cyto
Assay	**NanoBRET-based assays**	**NanoBRET-based assays**	**NanoBRET-based assays**
Measurement Mode	Dual Color Luminescence	Luminescence Multi Color	Luminescence Multi Color
Filter 1 / Wavelength Range 1	Blue1_NB	445-470 nm	445-470 nm
Integration time	300 ms	300 ms	300 ms
Filter 2 / Wavelength Range 2	Red_NB	610-635 nm	610-635 nm
Integration time	300 ms	300 ms	300 ms
Settle time	0 ms	0 ms	0 ms

SUPPORT.

Links.

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
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TECAN – WHO WE ARE.

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Founded in Switzerland in 1980, Tecan has manufacturing, research and development sites in both North America and Europe, and maintains a sales and service network in 52 countries. To date, Tecan has distributed approximately 20,000 microplate readers worldwide, and is committed to continuous technological improvements and compliance with the highest global quality standards.

Important information.

Tecan has not independently validated the methods described in the document with all possible sample types or analytical uses and is providing this example method as a convenience to users. Consistent with USP recommendations and good laboratory practice, the user must independently evaluate and validate: (a) the suitability of the method for their use, (b) their ability to process samples of their choosing following the method; and (c) their ability to proficiently perform the method in their facility with their personnel.

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