Cell-based assays

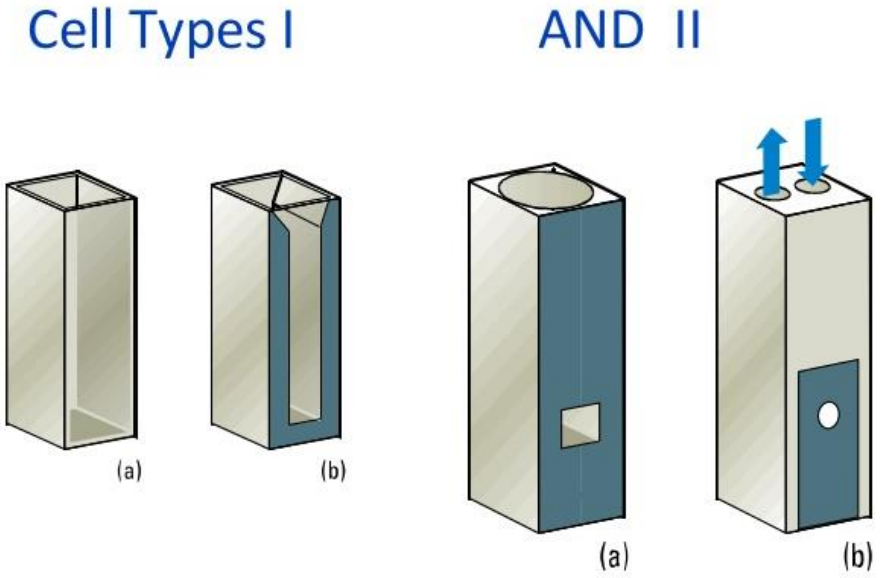
Colorimetric assays

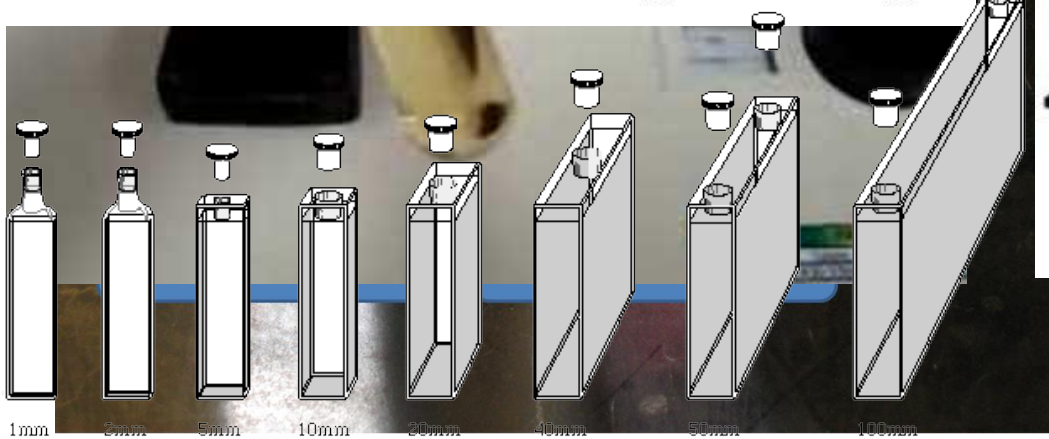
Prof. Dr. Andrei Leitao

Electromagnetic spectrum

http://pt.slideshare.net/suniu/spectrophotometry-16091660

Spectrophotometers





http://www.umich.edu/~chem125/softchalk/Exp2_Final_2/Exp2_Final_2_print.html
https://www.youtube.com/watch?v=Rem9KkolKBI
http://www.news-medical.net/Multiskan-GO-Microplate-Spectrophotometer-from-Thermo-Scientific
http://www.starnacells.com/d_cells_s/rect/T021.html
http://www.nanodrop.com/productnd2000overview.aspx

Inside the spectrophotometer

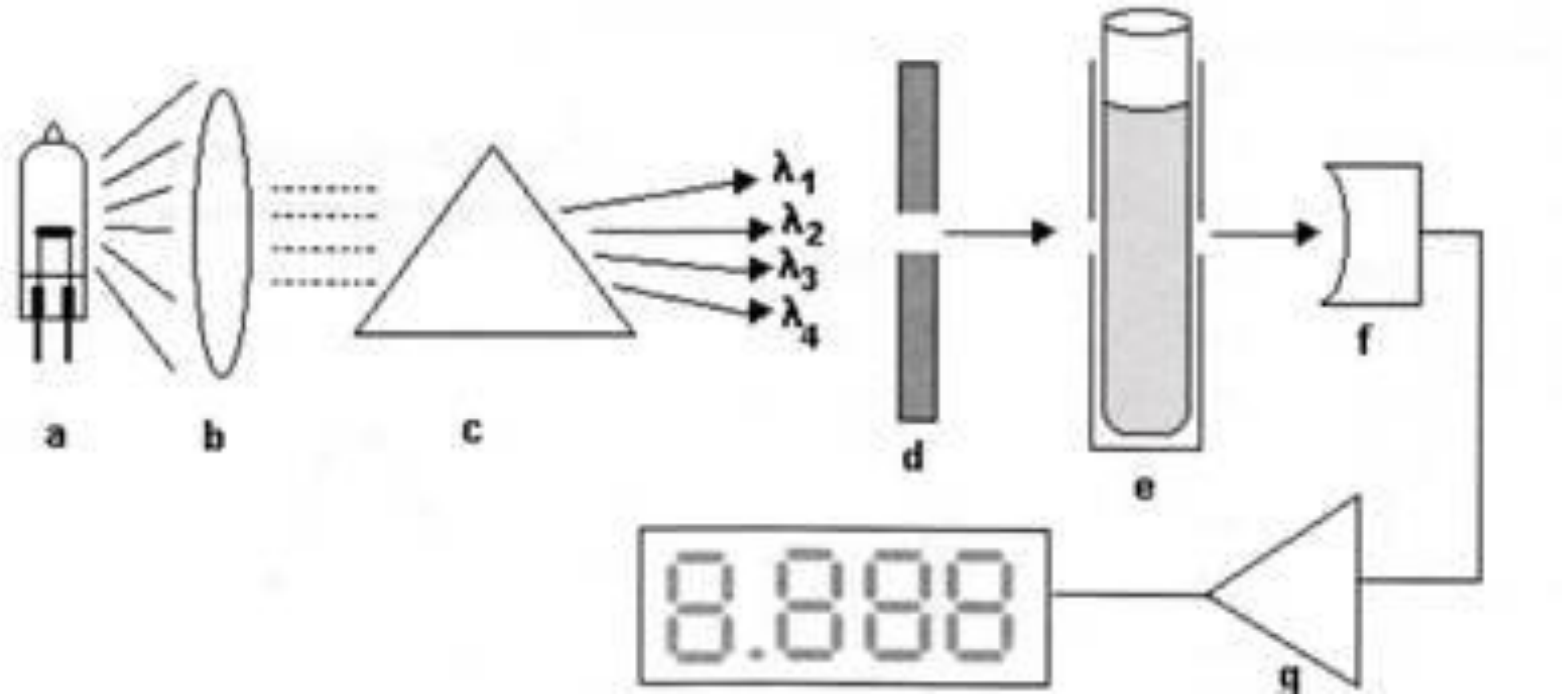


Figure. Optical scheme of the main spectrophotometer components.
(a) Light source, (b) Colimator, (c) Prism, (d) λ selector slit, (e) sample compartment with cubette, (f) photoellectric cell, (g) amplifier.

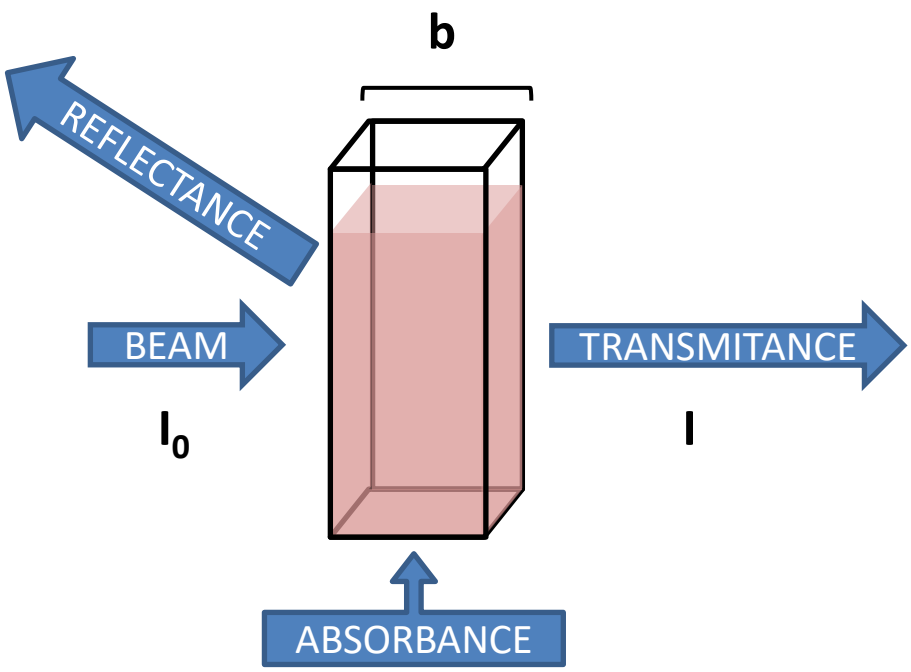
https://carriescsustan.edublogs.org/2015/12/10/building-a-spectrophotometer/
http://www.ufrgs.br/leo/site_espec/componentes.html

Colorimetric assays –basic principles

Lambert - Beer's Law

$$A = log\frac{I_0}{I} = \varepsilon bc$$

ε = molar absorptivity
b = sample's path lenght
c = concentration



Colorimetric assays –basic principles

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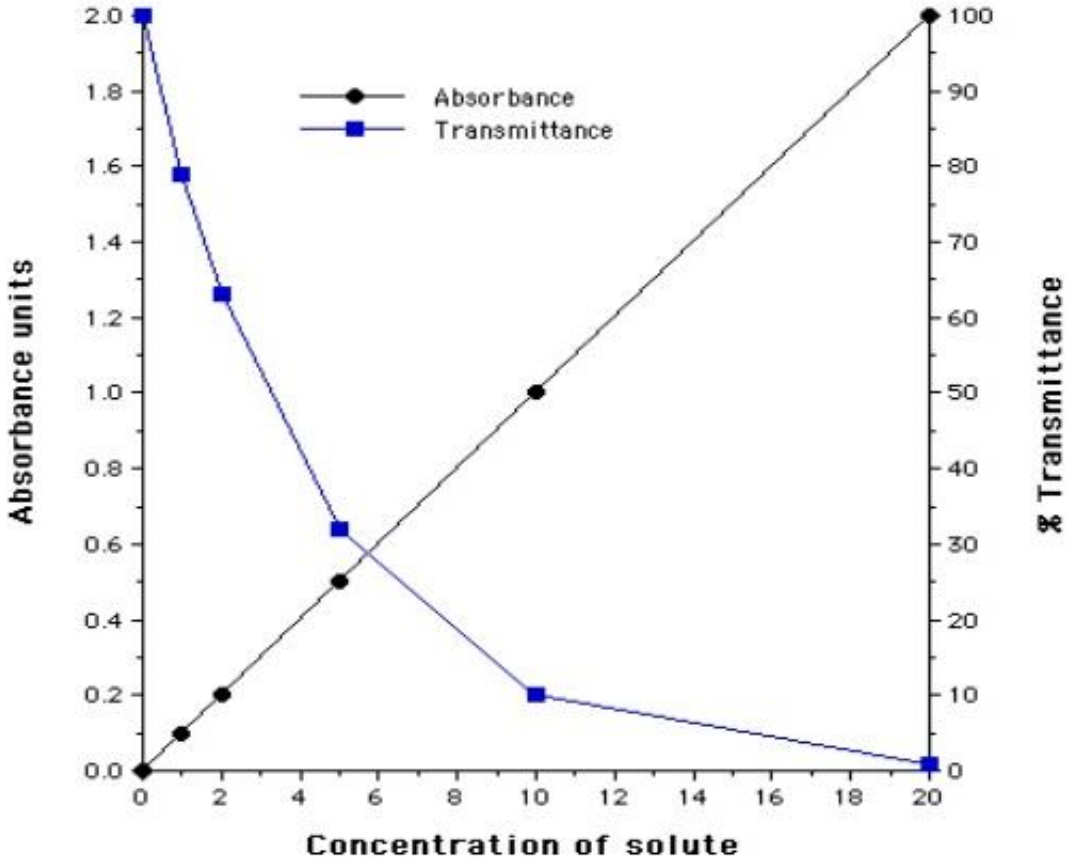
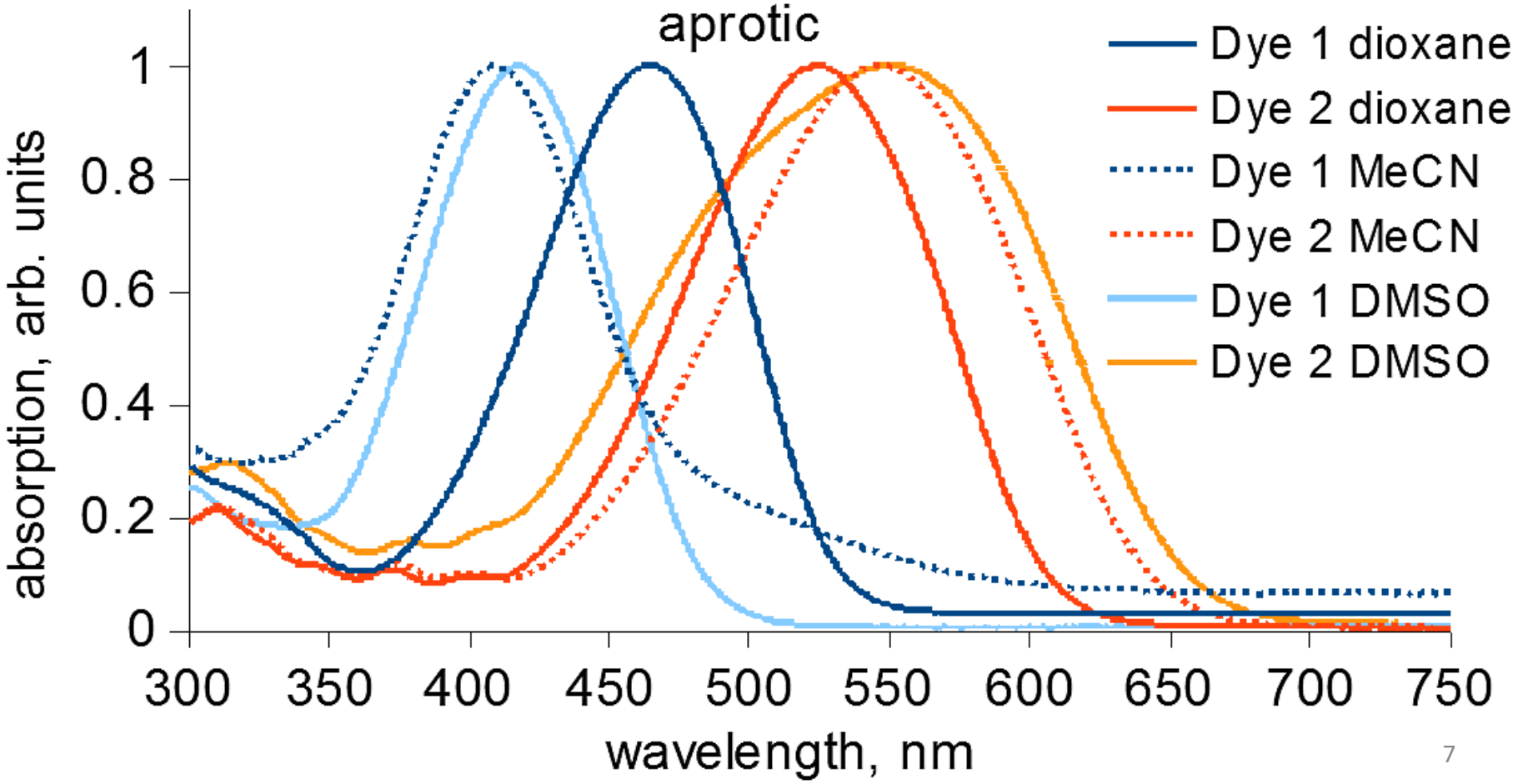


FIGURE 13-4 Deviations from Beer's law with polychromatic radiation. The absorber has the indicated molar absorptivities at the two wavelengths λ′ and λ″.

Solvatochromism

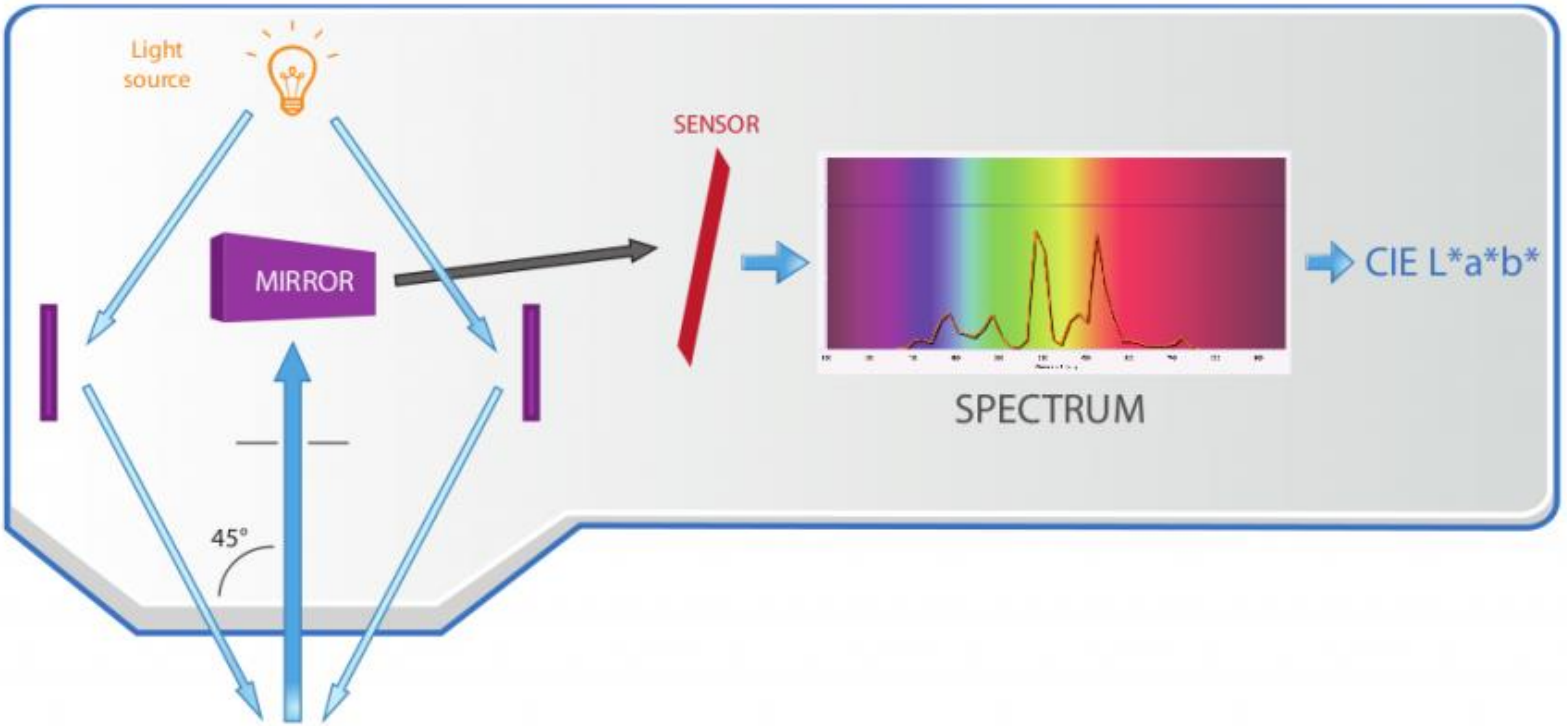
hypsochromic shift **bathochromic shift**



The Spectrophotometer

It is a colour measurement device that provides accurate readings about the current conditions of the press or computer screen

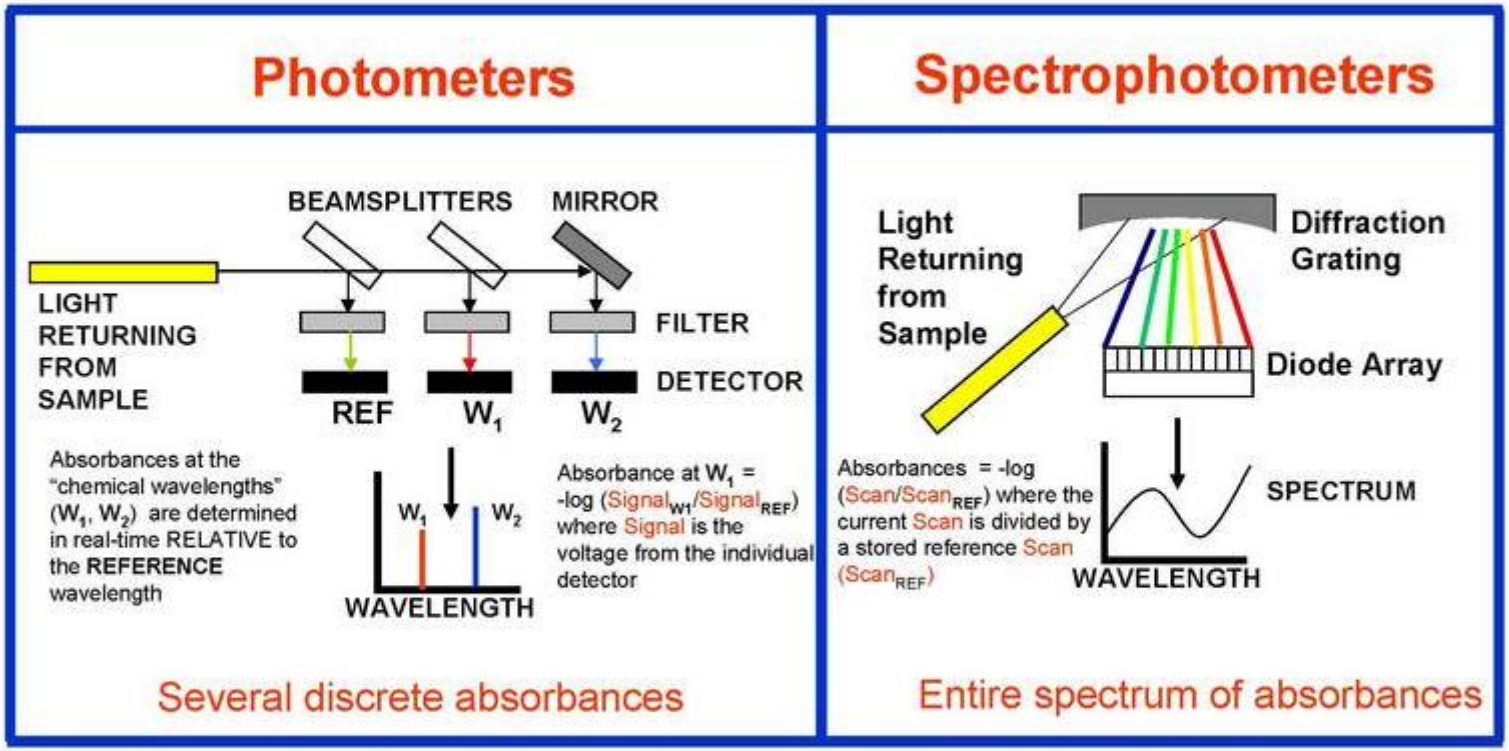
How does it work ?



CALDERA

http://blog.caldera.com/spectrophotometers-do-not-measure-colors/

How They Work

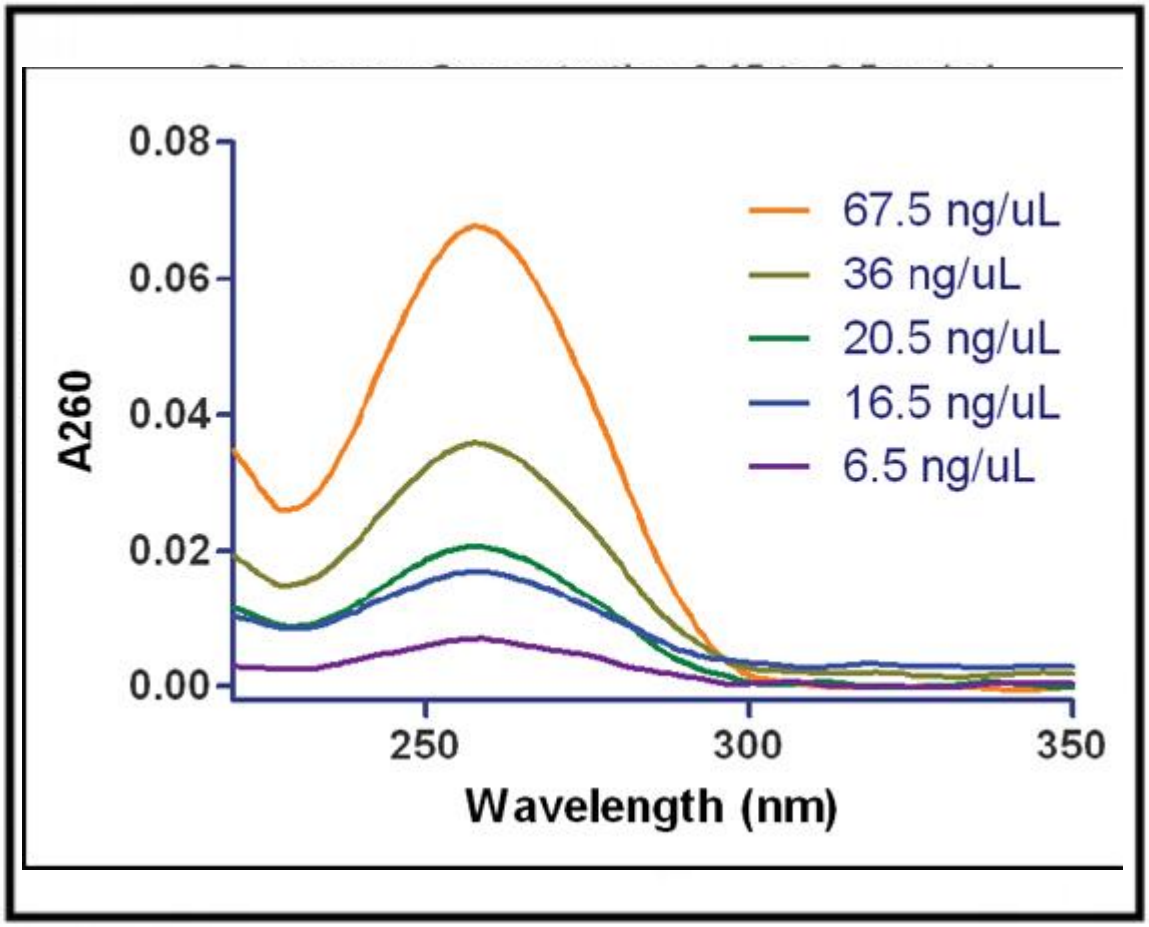


Note: there are other methods, such as rotating filter wheel photometers and FT-NIR or rotating grating spectrophotometers

Guided Wave
Process Analytical Systems

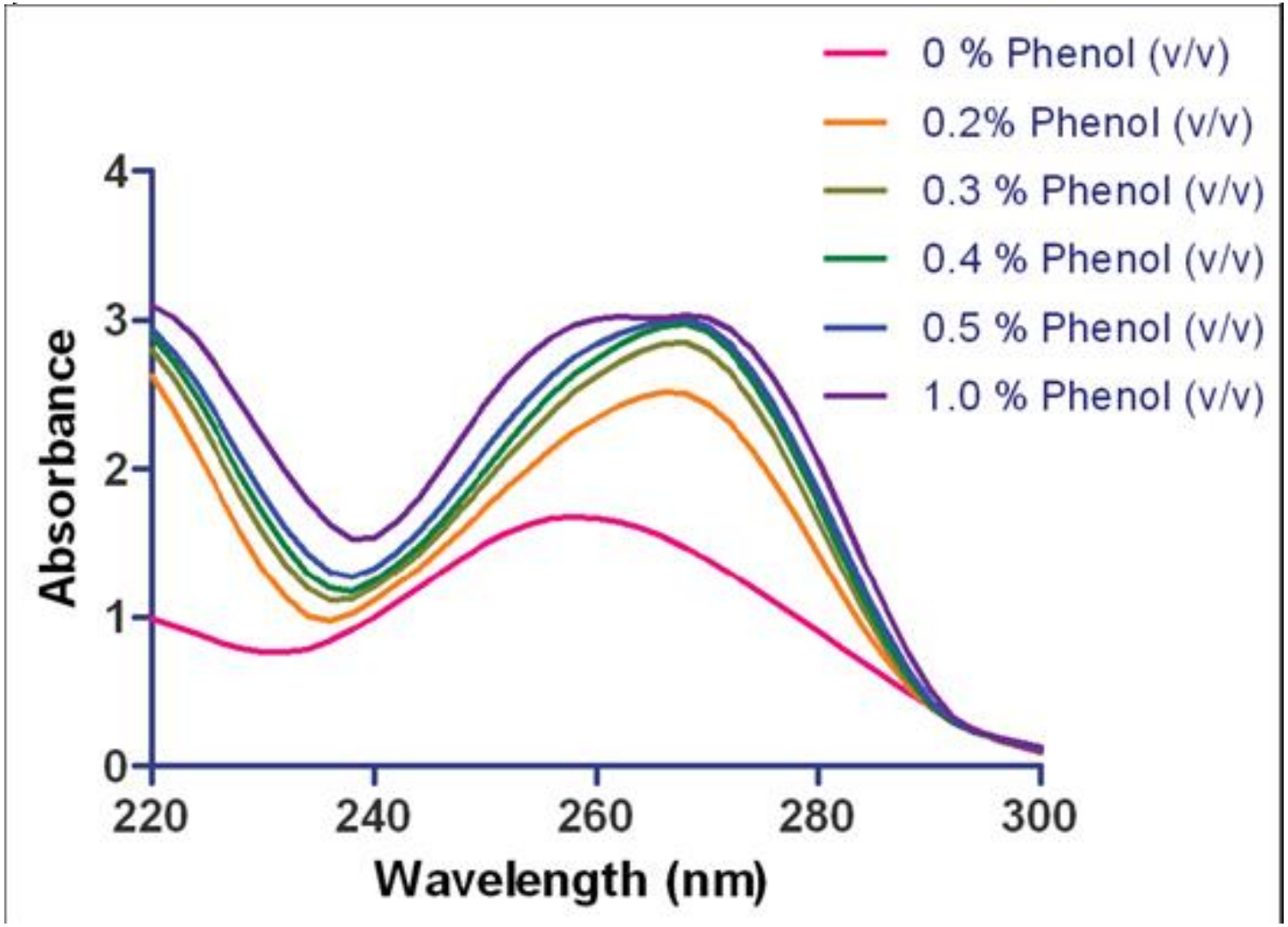
http://www.guided-wave.com/gwi-document-pages/photometer-vs-spectrometer.html

Assays – DNA and proteins



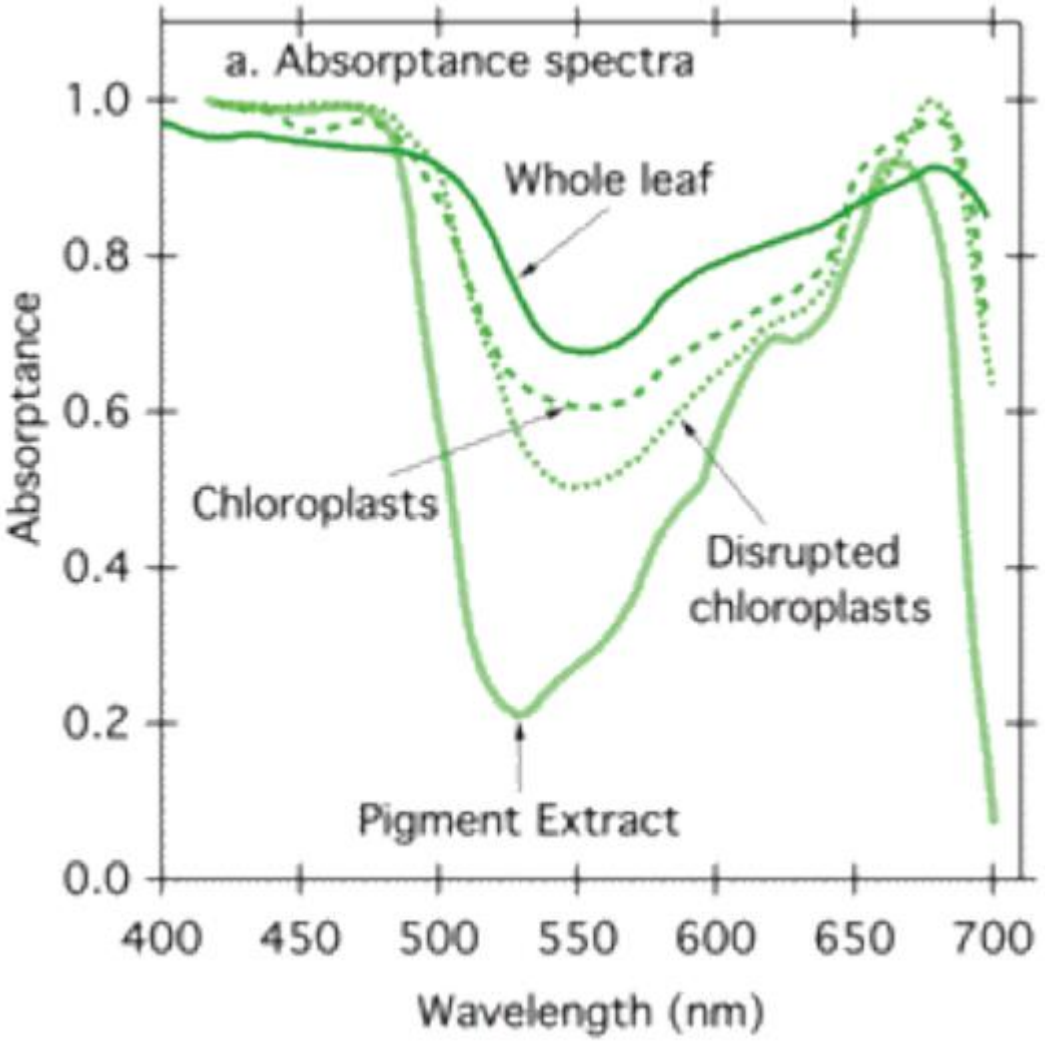
http://oceanoptics.com/measuring-dna-absorbance-sts-uv-microspectrometer/
http://www.biotek.com/resources/articles/micro-volume-purity-assessment-nucleid-acids.html

Assays – DNA and proteins



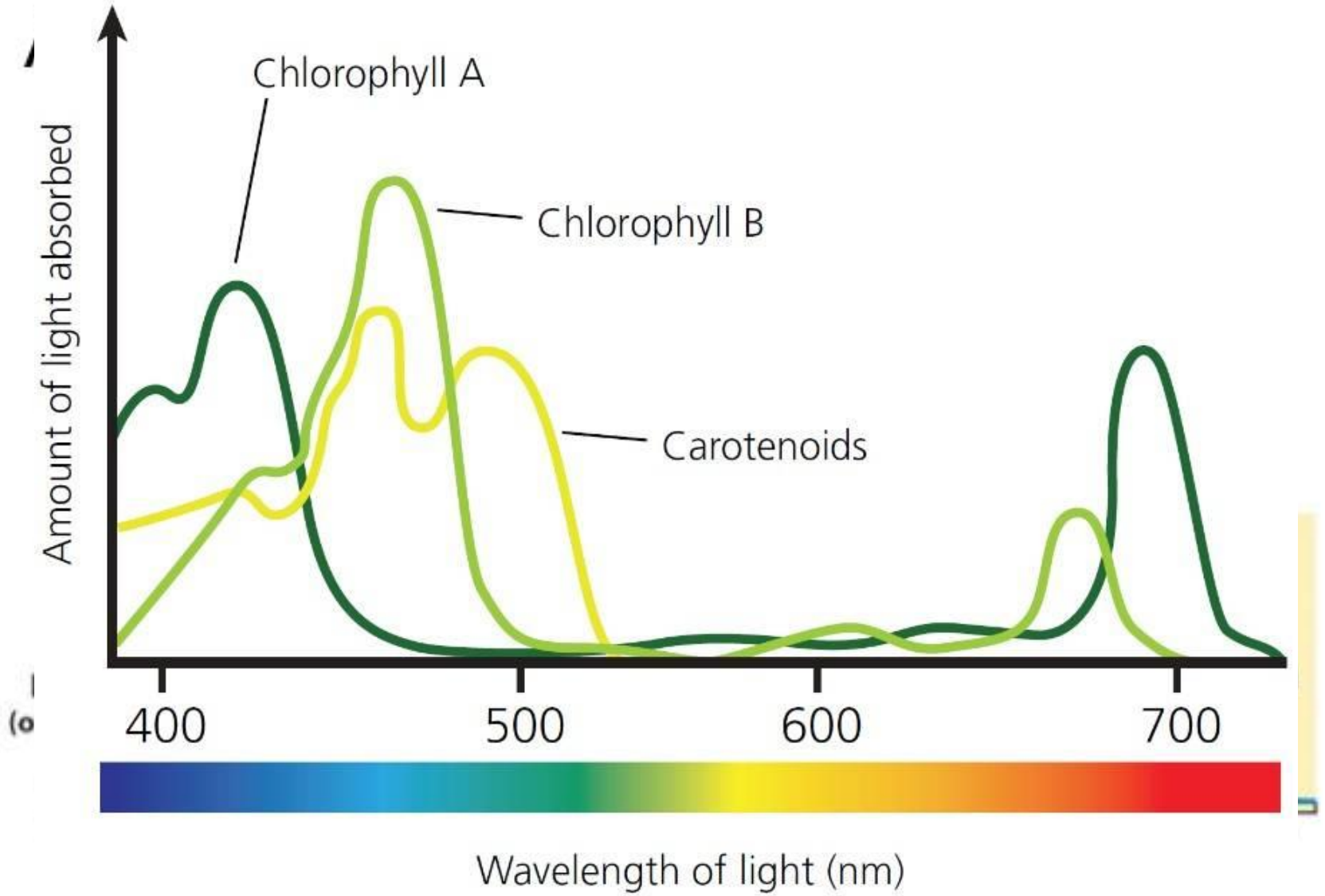
http://www.biotek.com/resources/articles/micro-volume-purity-assessment-nucleid-acids.html

Mixing everything...



https://www.heliospectra.com/blog/spectrum-101-absorption-spectra-versus-action-spectra

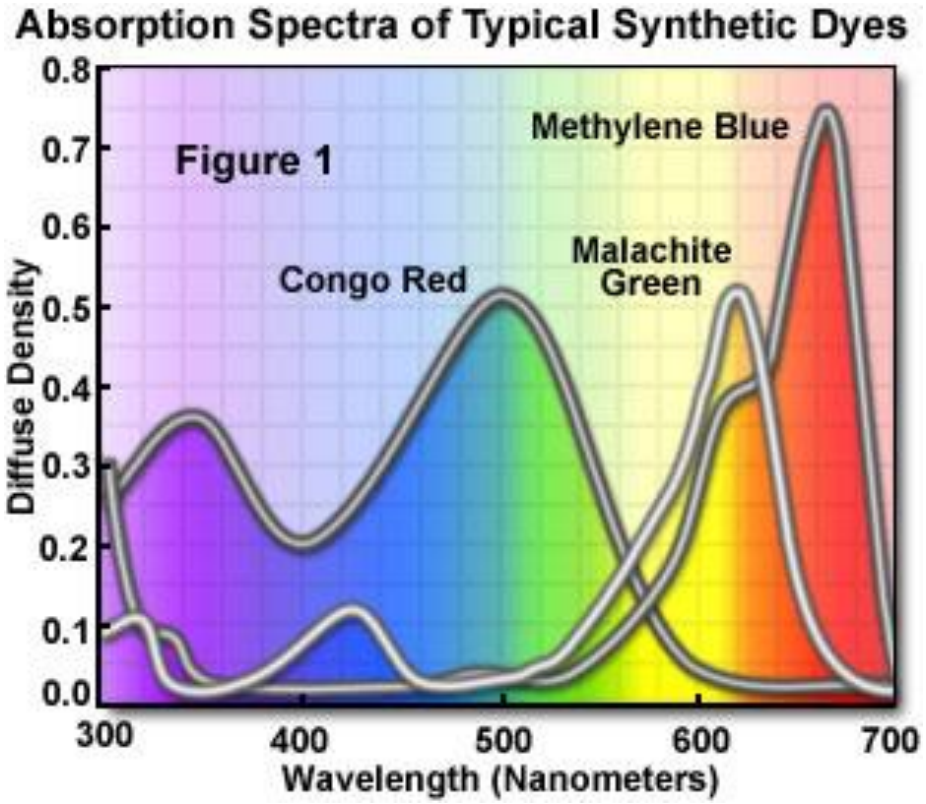
Chemicals – some examples

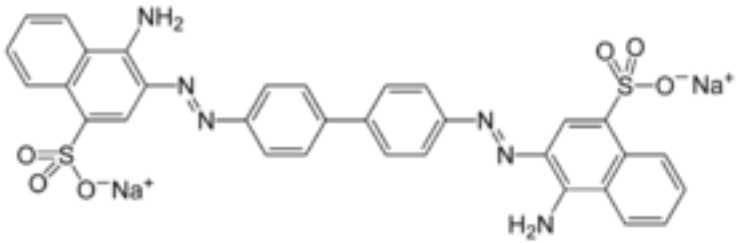


http://pt.slideshare.net/codyalantaylor/spectrophotometry-lecture

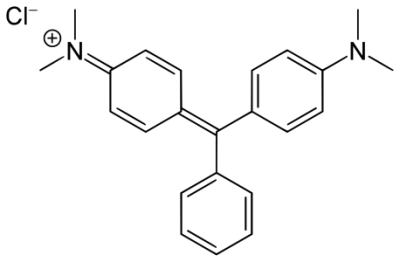
https://www.heliospectra.com/blog/spectrum-101-absorption-spectra-versus-action-spectra

Molecular absorption of some biological stains

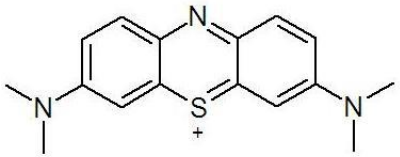




congo red

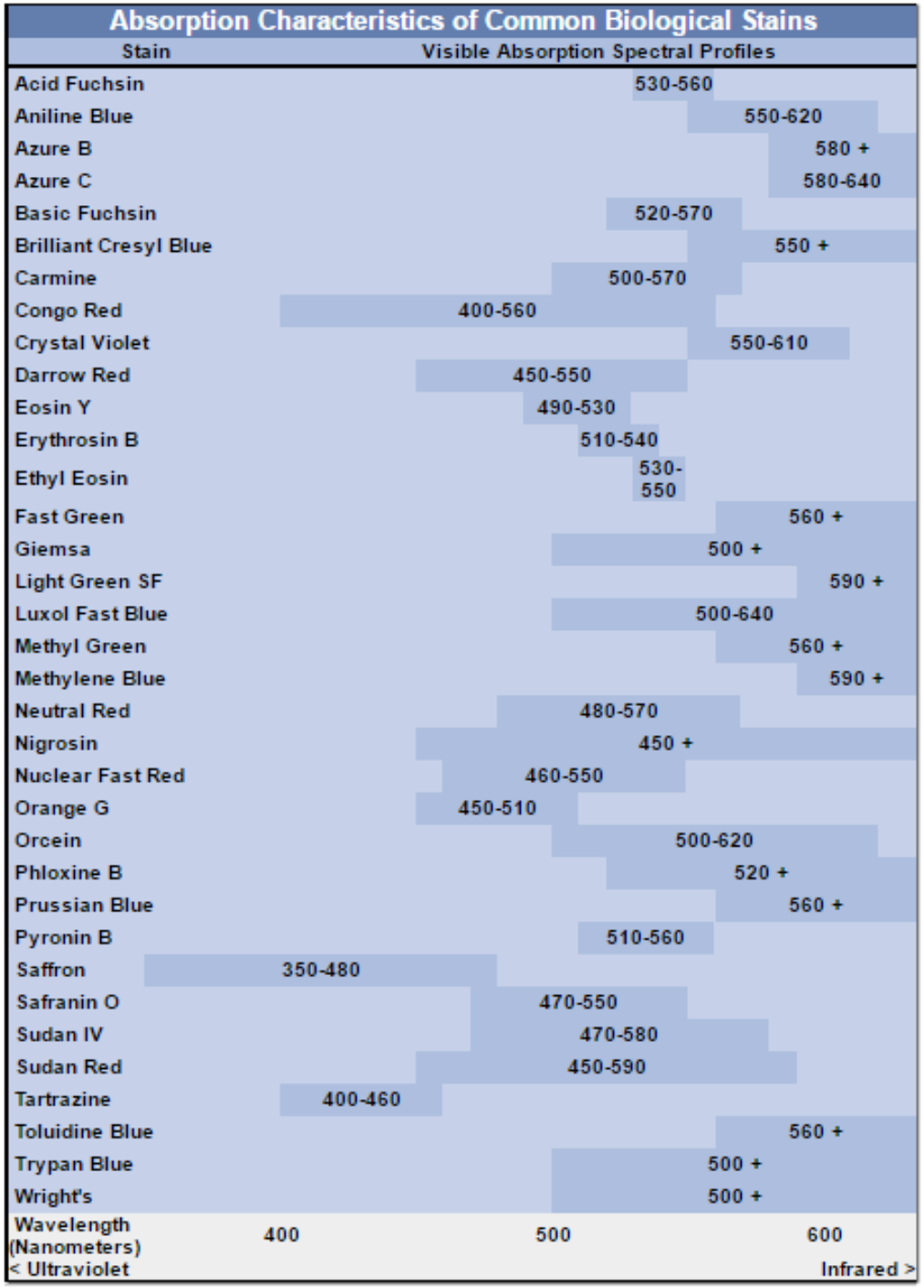


malachite green



methylene blue

https://micro.magnet.fsu.edu/primer/photomicrography/bwstainchart.html



https://micro.magnet.fsu.edu/primer/photomicrography/bwstainchart.html

Colorimetric assays

- ✓ Tetrazolium salts
- ✓ Trypan blue
- ✓ Sulforhodamine B
- ✓ Neutral Red
- ✓ Resarzurin - Alamar blue

...

Tetrazolium salts

monotetrazolium salts are reduced by NAD(P)H-dependent oxidoreductases and dehydrogenases of metabolically active cells.

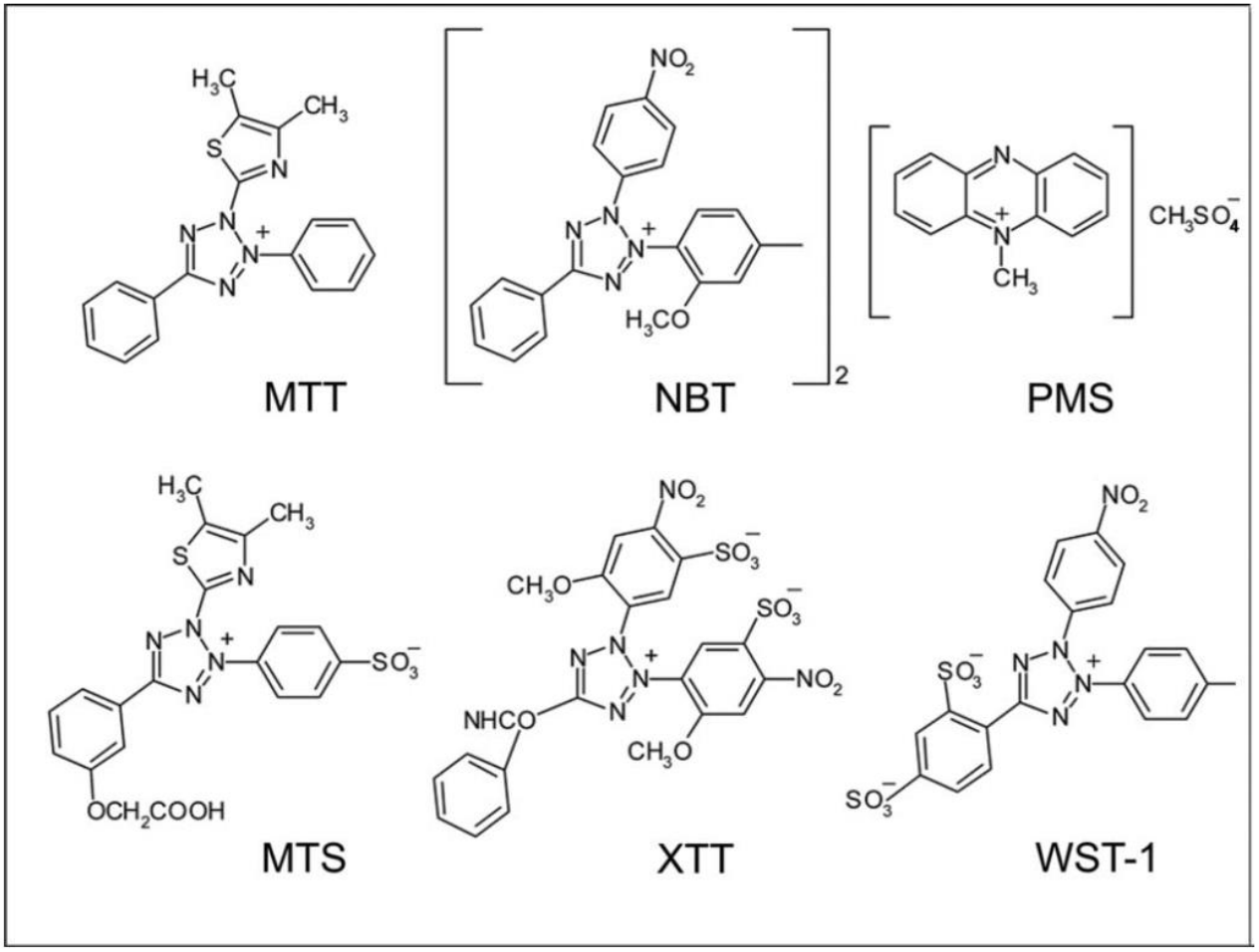


Fig. 1. Chemical structures of selected tetrazolium salts and of the intermediate electron acceptor, phenazine methosulfate (PMS).

Berridge, M.V.; Herst, P.M.; Tan, A.S.

Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction.

Biotechnol. Ann. Rev. **2005**, *11*, 127-152.

Tetrazolium salts

reduction rates

MTT > MTS > XTT = WST-1

Superoxide does not cross de cell membrane

Studies also showed that MTT reduction does not correlate with DNA synthesis. 24h and above, it has some correlation with H^3-thymidine (for DNA incorporation).

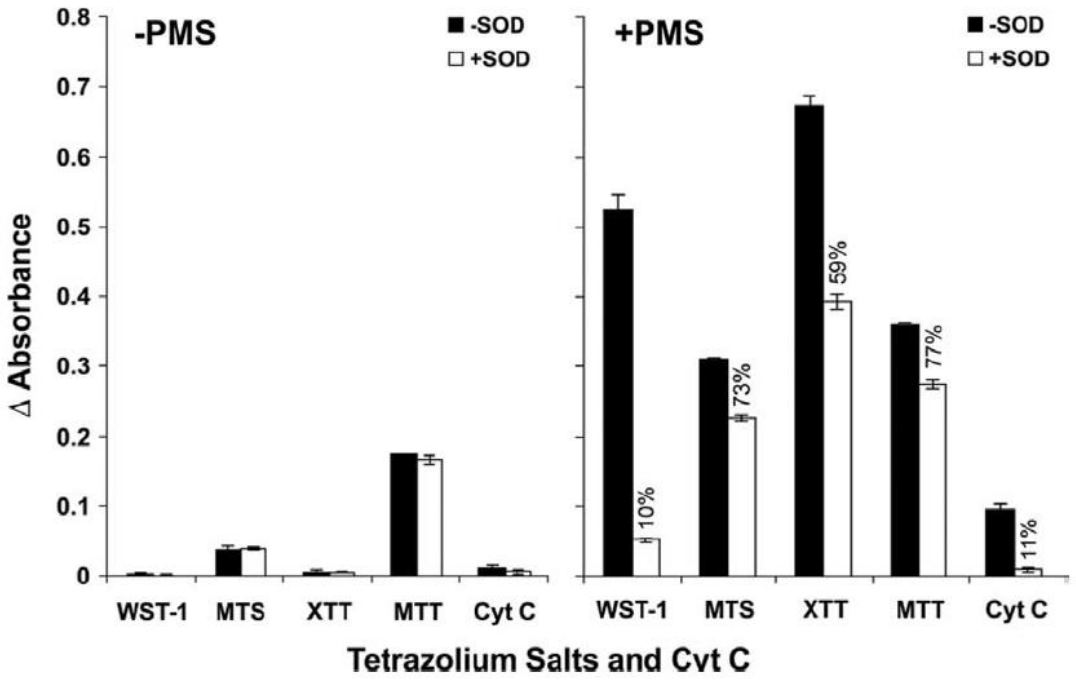
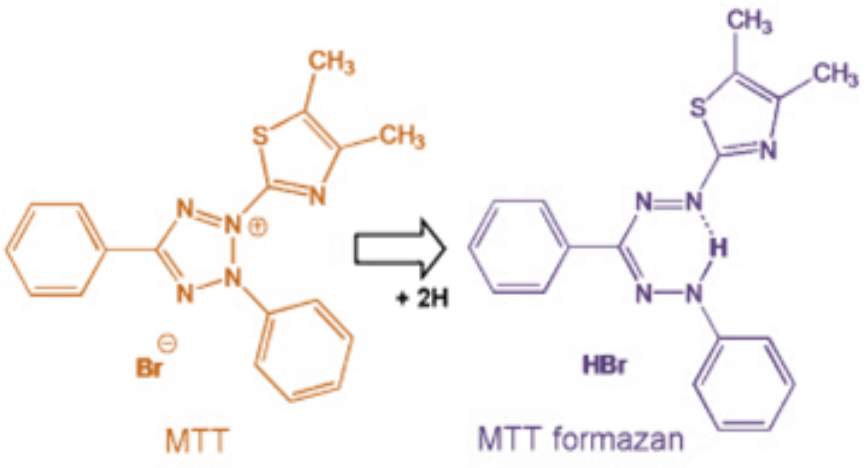
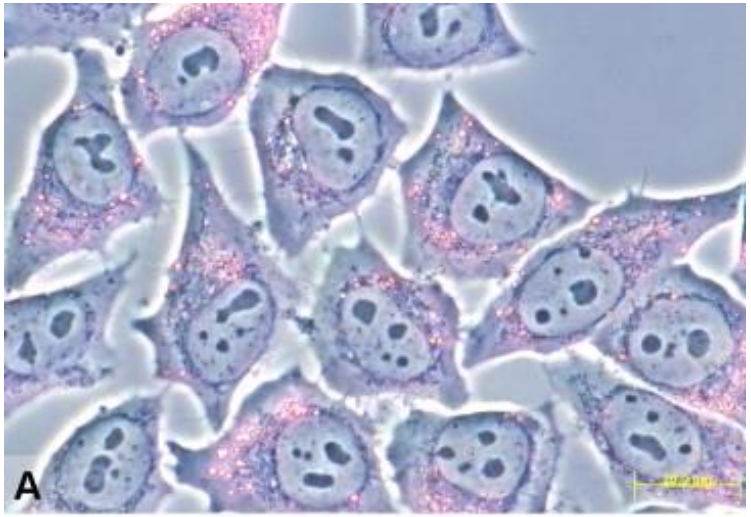
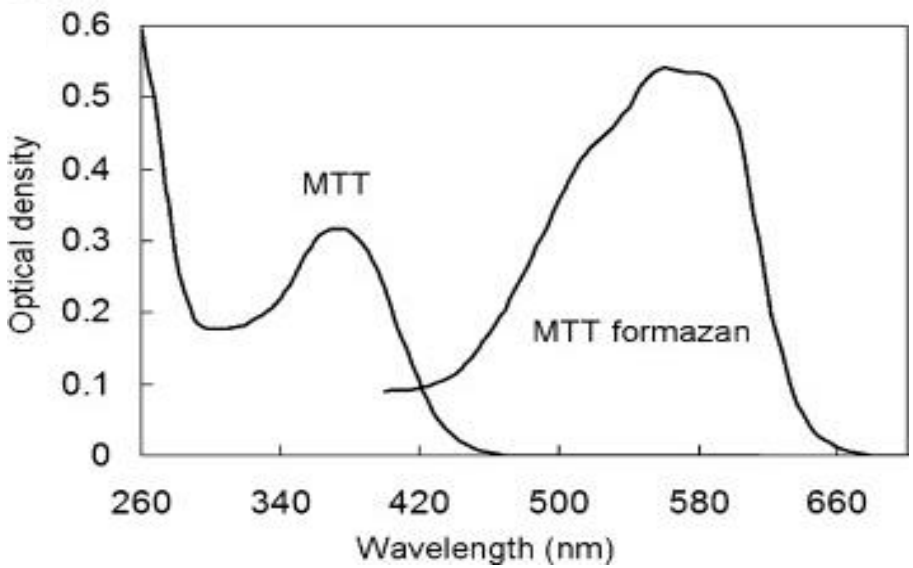


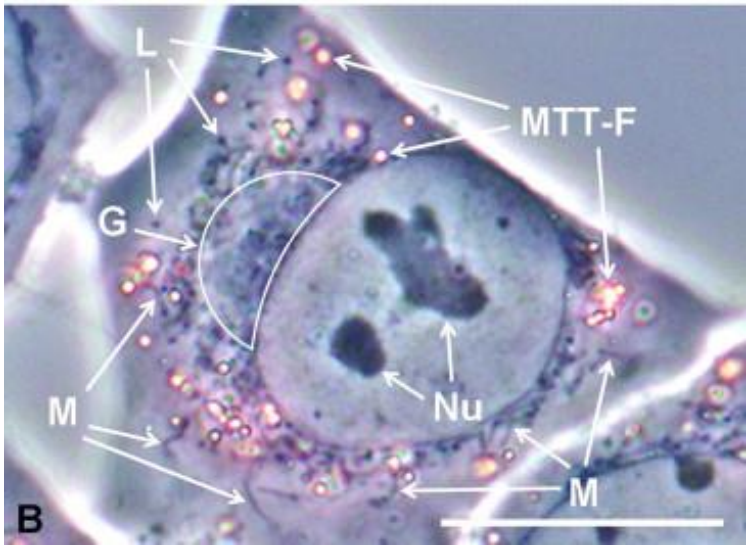
Fig. 3. Comparison of cellular tetrazolium dye reduction in the presence and absence of mPMS and SOD. Human T-lymphoblastic Jurkat cells (2–3 × 10^4 per microplate well) were incubated for 1 h with WST-1 (400 μg/ml), MTS (313 μg/ml), XTT (313 μg/ml), MTT (500 μg/ml) or ferricytochrome *c* (80 μM) in the presence and absence of mPMS (20 μM) and SOD (20 μg/ml). Absorbance was measured in a microplate reader at 450 nm for WST-1, MTS and XTT, 570nm for MTT and 550 nm for cytochrome *c*. SOD inhibition is presented as % control. Results are presented as the mean of duplicate determinations ± standard error.

MTT









Stockert, J. C.; Blázquez-Castro, A.; Cañete, M.; Horobin, R. W.; Villanueva, A. MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. *Acta Histochemica*, **2012**, *114*, 785–796

MTT

Table 1
Interfering factors

Factor	Type of interference	Solution
Glucose	Lack of glucose lowers MTT production through decreased glycolysis	Do not use exhausted medium (change it few hours before assay)
Protein precipitation	Organic solvents may cause precipitation of serum proteins, which interferes with the reading	Use different solvent
Phenol red	Phenol red interferes with reading, its absorbance is pH-dependent	Use acidified solvent or medium without phenol red
Incomplete solubilization	Formazan crystals do not dissolve, and this lowers the sensitivity of the assay	Increase shaking intensity and/or extraction time
Confluency	(Over)confluent cells may lower their metabolic rate, resulting in underestimation of cell number	Use lower initial seeding density if possible
Metabolic rate	The metabolic rate of the cells is changed by the treatment	Use standard curve for each treatment, or find alternative cell counting method

WST

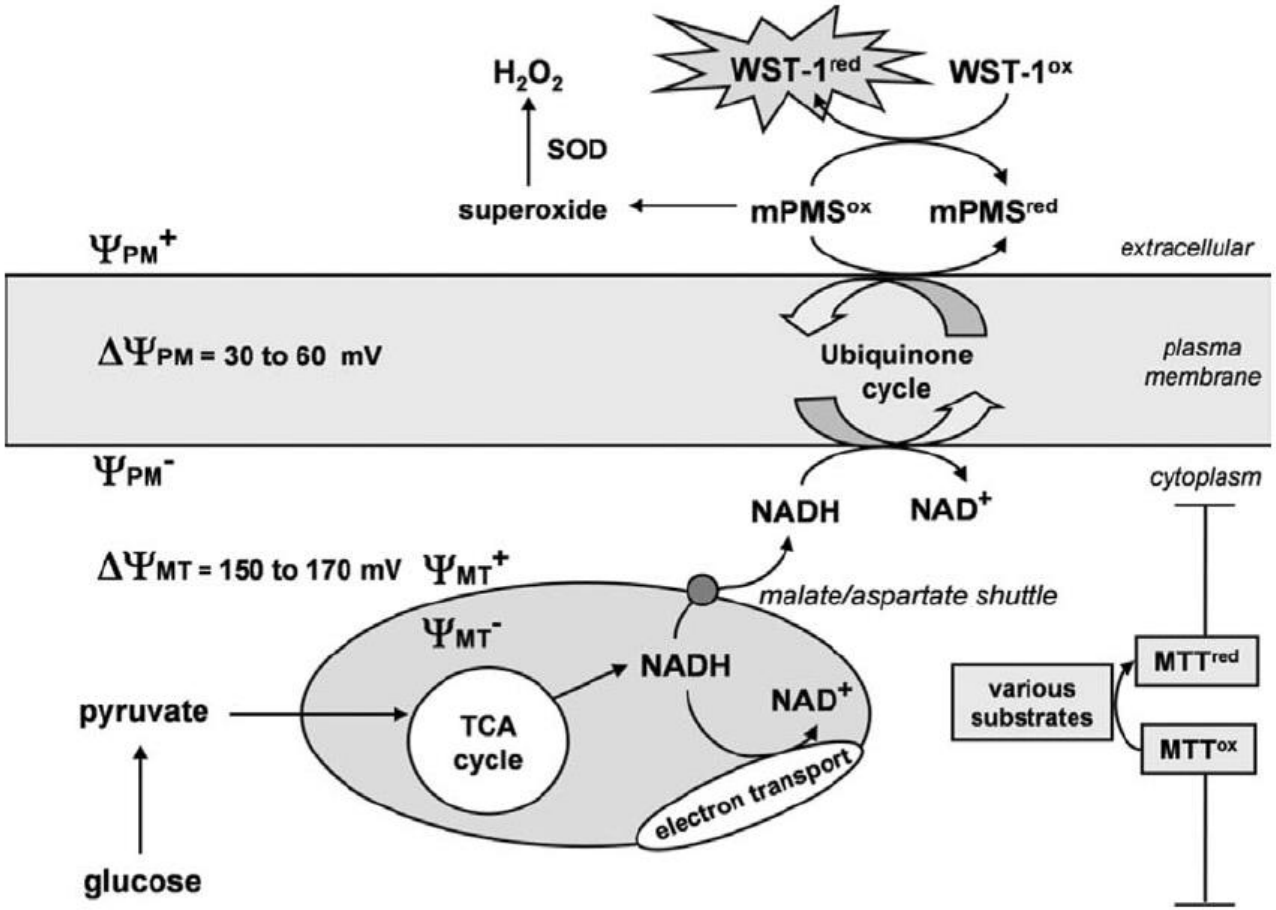
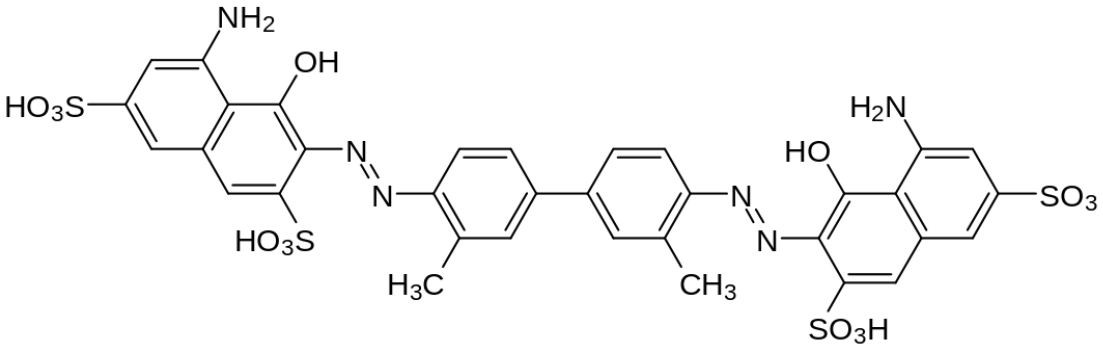
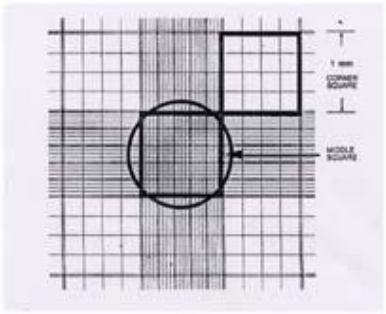


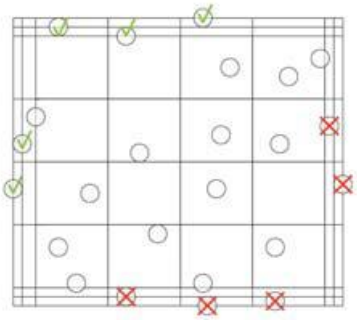
Fig. 2. Schematic representation of the proposed mechanisms of cellular reduction of MTT and WST-1. Whereas MTT is reduced by a variety of intracellular reductants, most notably NADH, WST-1 is reduced by trans-plasma membrane electron transport via the electron carrier, 1-methoxyPMS, in which case the cellular reductant is NADH derived mainly from the mitochondrial TCA cycle. The plasma membrane potential, which is proposed to be the major cellular determinant of tetrazolium dye uptake is also depicted.

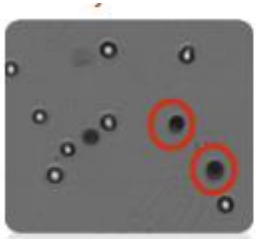
Trypan blue

Colorimetric method for the analysis of cell viability.
Incorporation of the dye means cell death due to membrane discruption.

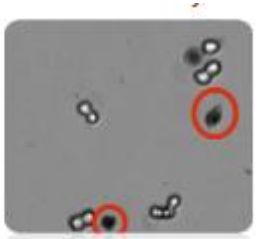




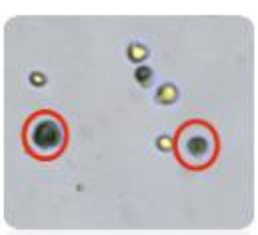




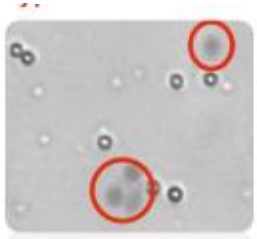
Jurkat cells imaged by Cellometer Vision



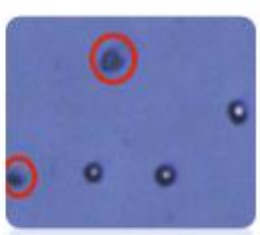
CHO cells imaged by Cellometer Vision



HeLa cells imaged by Cellometer Auto T4



Splenocytes imaged by Cellometer Auto 2000

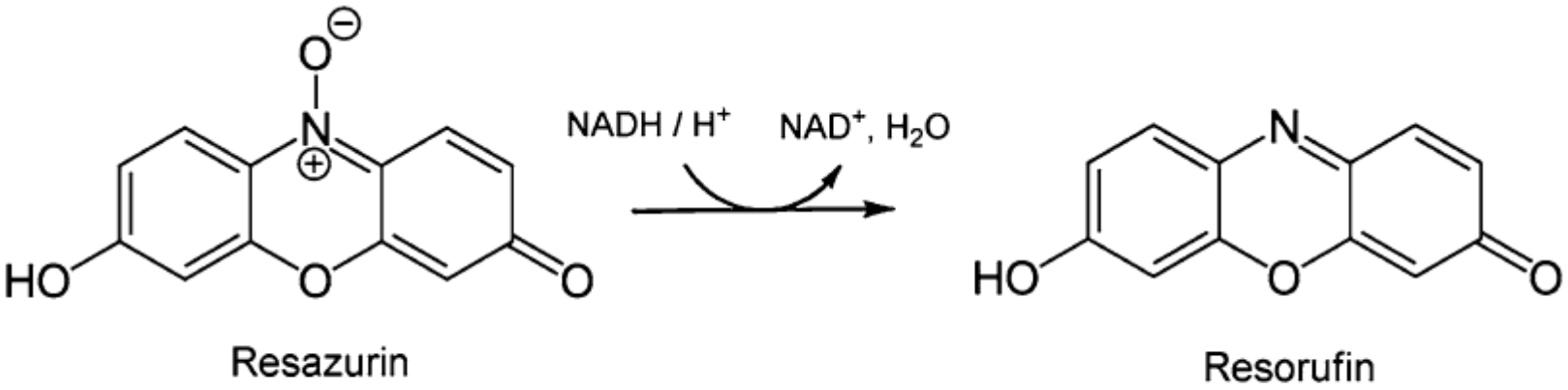


Jurkat cells imaged by Cellometer Auto 1000

More recently: use of this dye as a fluorescent marker.

http://www.nexcelom.com/Applications/measure-cell-viability-using-trypan-blue-or-AOPI.php
Brito-Melo, G.E.A. *et al.* Trypan blue exclusion assay by flow cytometry. *Braz. J. Med. Biol. Res.* **2014**, *47*, 307-315

Resarzurin



Resazurin

Resorufin

Mechanism of reduction of resazurin to resorufin.

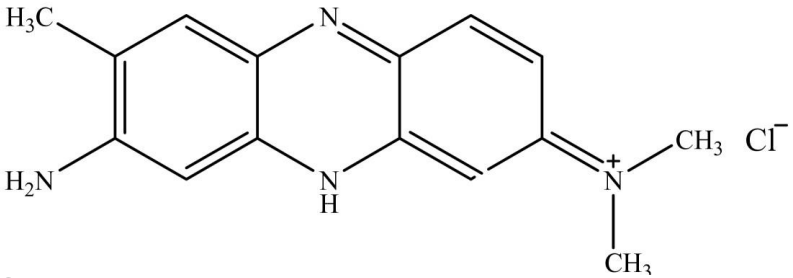
Ewa M. Czekanska. Assessment of Cell Proliferation with Resazurin-Based Fluorescent Dye. Chapter 5. Martin J. Stoddart (ed.), *Mammalian Cell Viability: Methods and Protocols*, Methods in Molecular Biology, vol. 740, DOI 10.1007/978-1-61779-108-6_5, © Springer Science+Business Media, LLC 2011

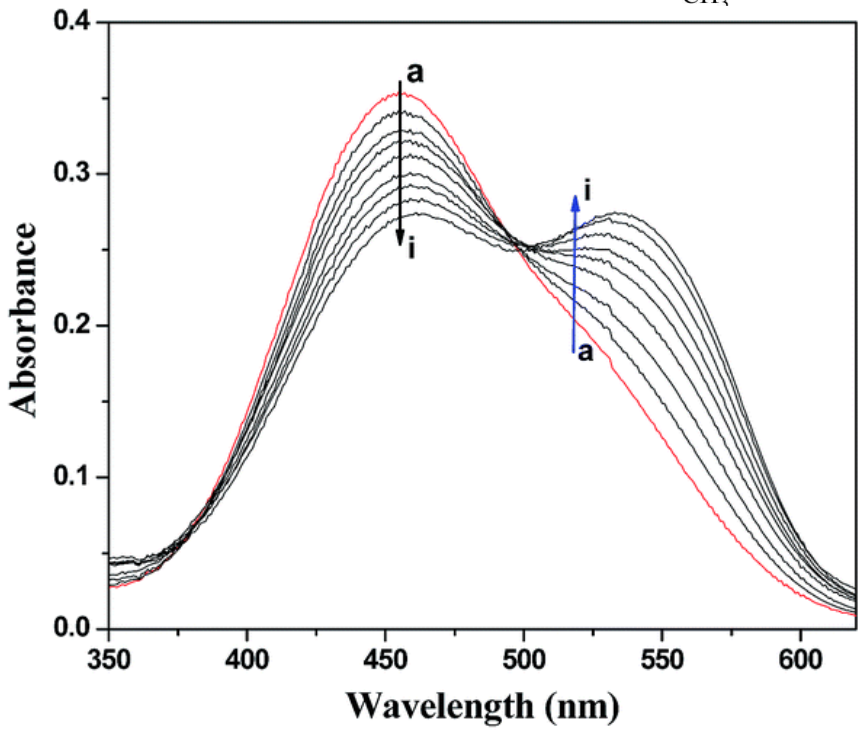
Resarzurin- Alamar blue

Table 1
The recommended conditions for the Alamar Blue assay in 96-well plate for normal and cancer cell lines. Reprinted from (14) with permission from Elsevier

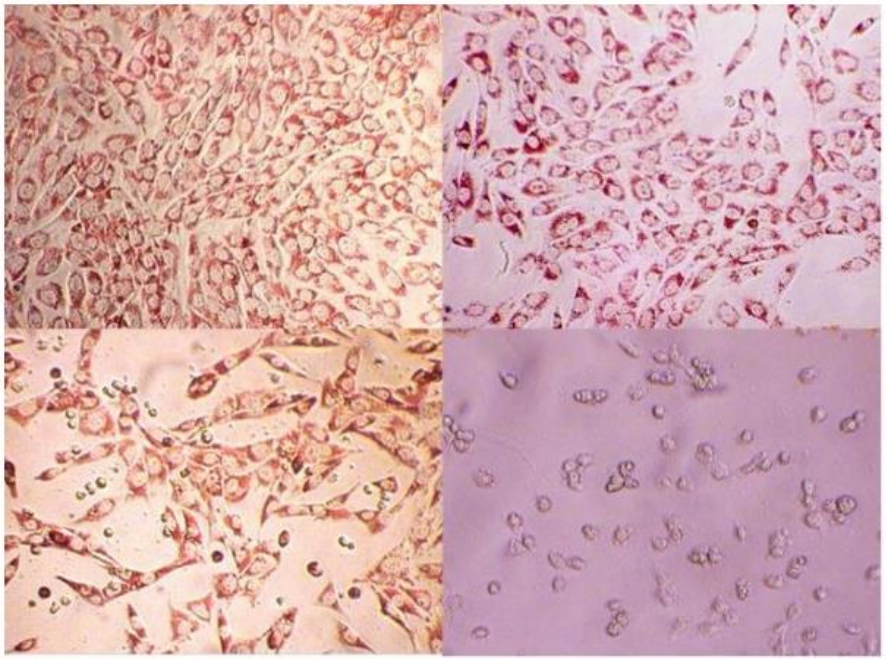
Cell line	Linear range (cell number × 10^4)	Optimal AB concentration (%)	Optimal incubation time (h)
Normal cell lines			
BALB/3T3	0.05–2	4	3
CHO-K1	0.05–3	10	3
NJTIDF 4054	0.05–2	4	3
Ovarian cancer cell lines			
2008	0.05–3	4	1
IGROV-1	0.05–3	10	1
OVCAR-3	0.05–3	4	3
SK-OV-3	0.05–3	4	3
Leukaemia cell lines			
HL-60	0.5–5	10	6
K-562	0.5–5	10	6
MOLT-4	0.5–5	10	6

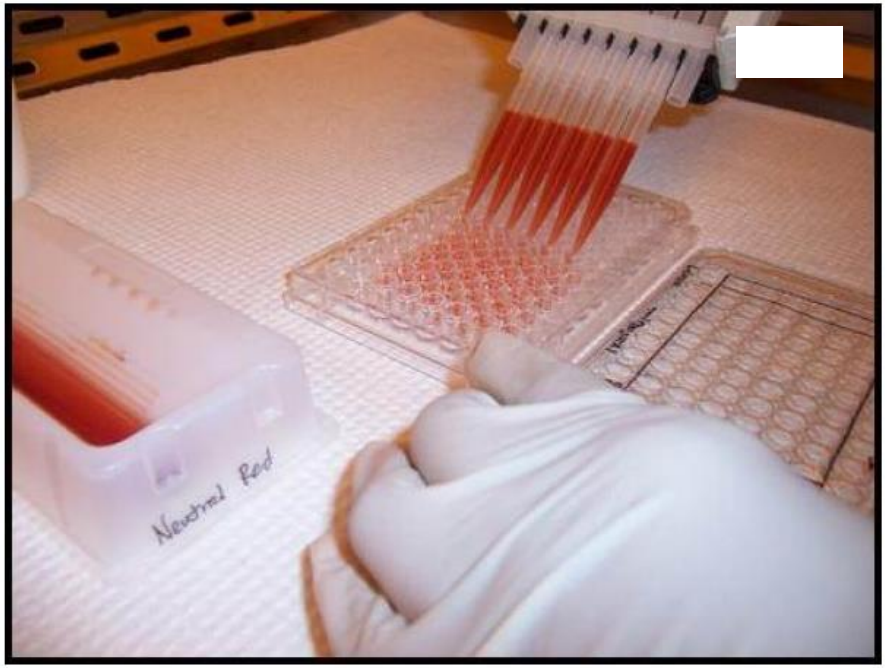
Neutral red





UV absorption spectra of NR in the presence of DNA at pH 7.4 and room temperature. [NR] = 20 μM., [DNA] = 0–52,5 μM.
Ref: ***Med. Chem. Commun.***, 2015,**6**, 222-229

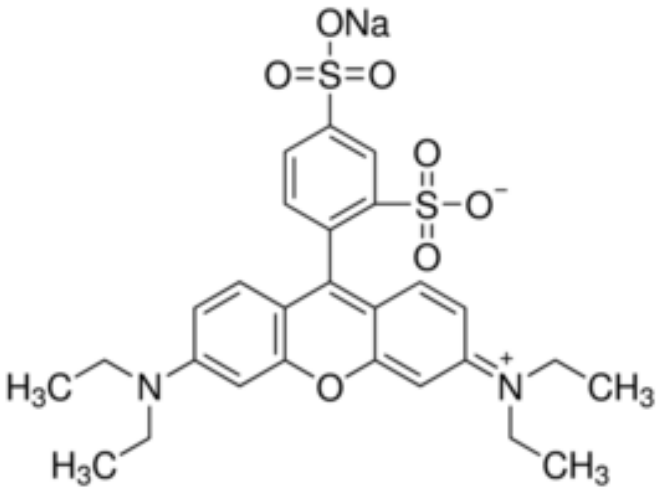


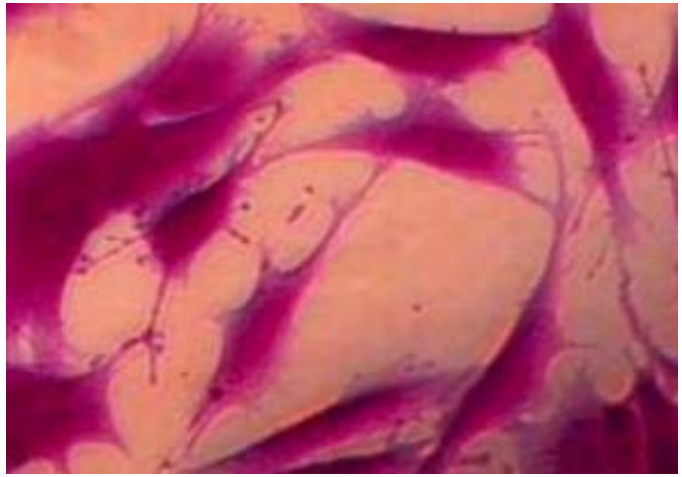


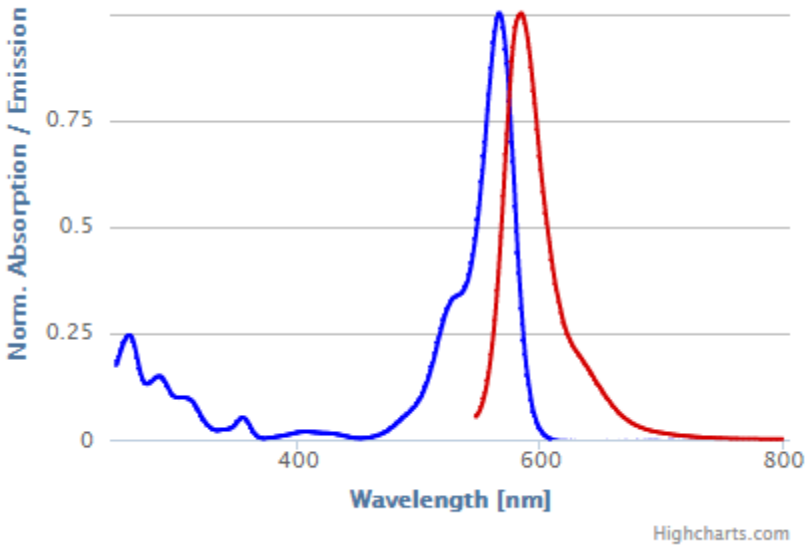
Neutral Red Addition: Neutral red is added to the 96-well plate.

http://w2ww.iivs.org/home/scientific-services/laboratory-services/systemic-tox/nhek-cytotoxicity/step-by-step

Sulforhodamine B







Overview

Absorption λ_{max}

566 nm, 355 nm

Emission λ_{max}

584 nm

Solvent

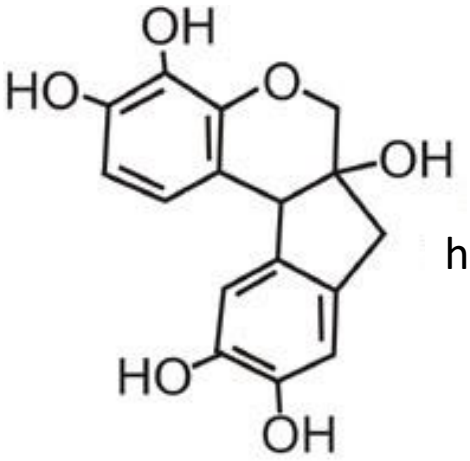
water

Molar Abs. Coefficient

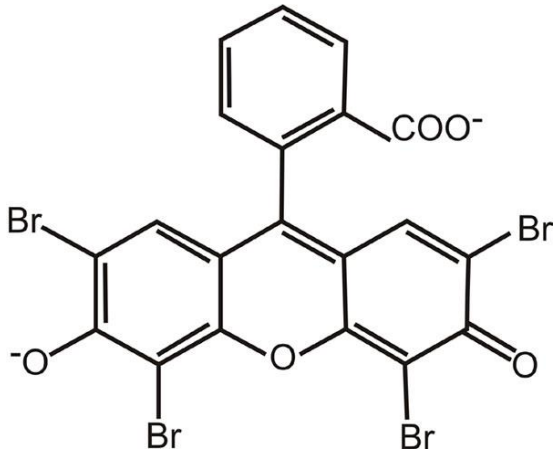
-

http://www.fluorophores.tugraz.at/
http://www.aniara.com/

Hematoxylin-eosin



hematoxylin



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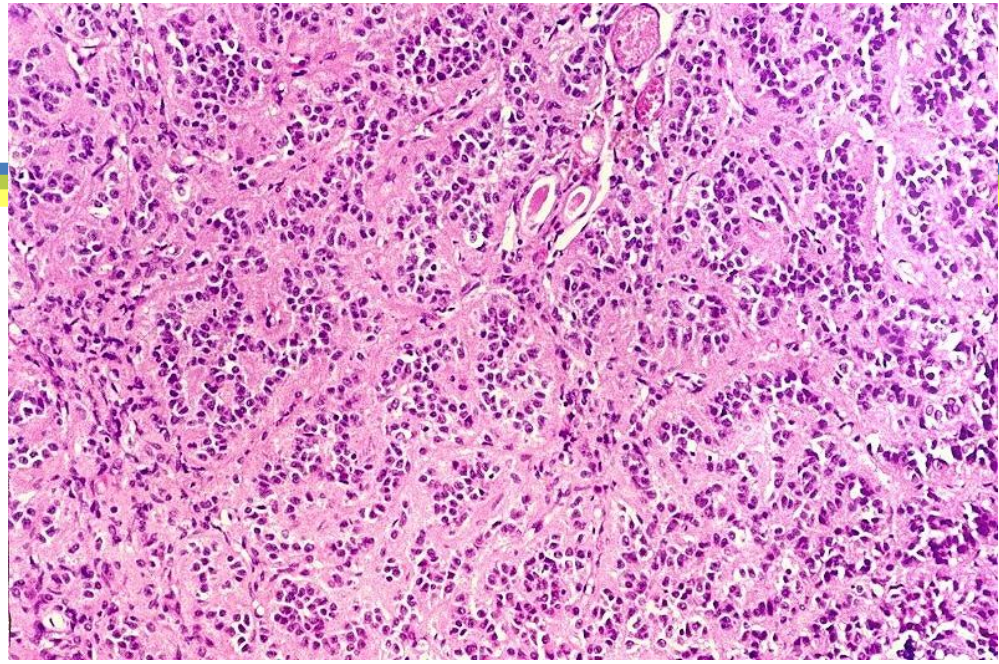

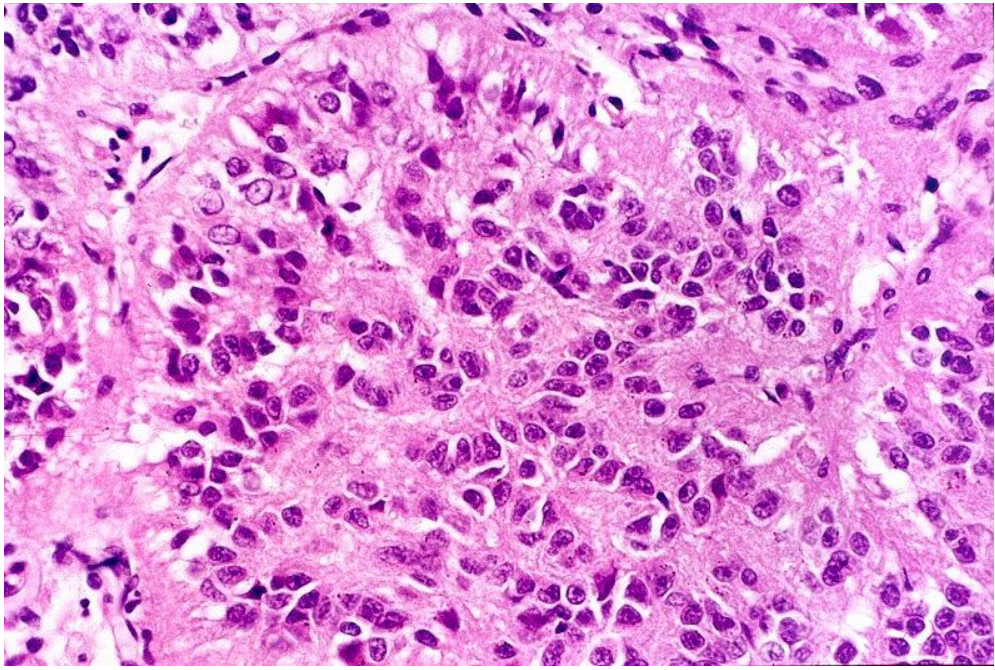

http://anatpat.unicamp.br/bineutupineal.html

Label-free technologies

Impedance

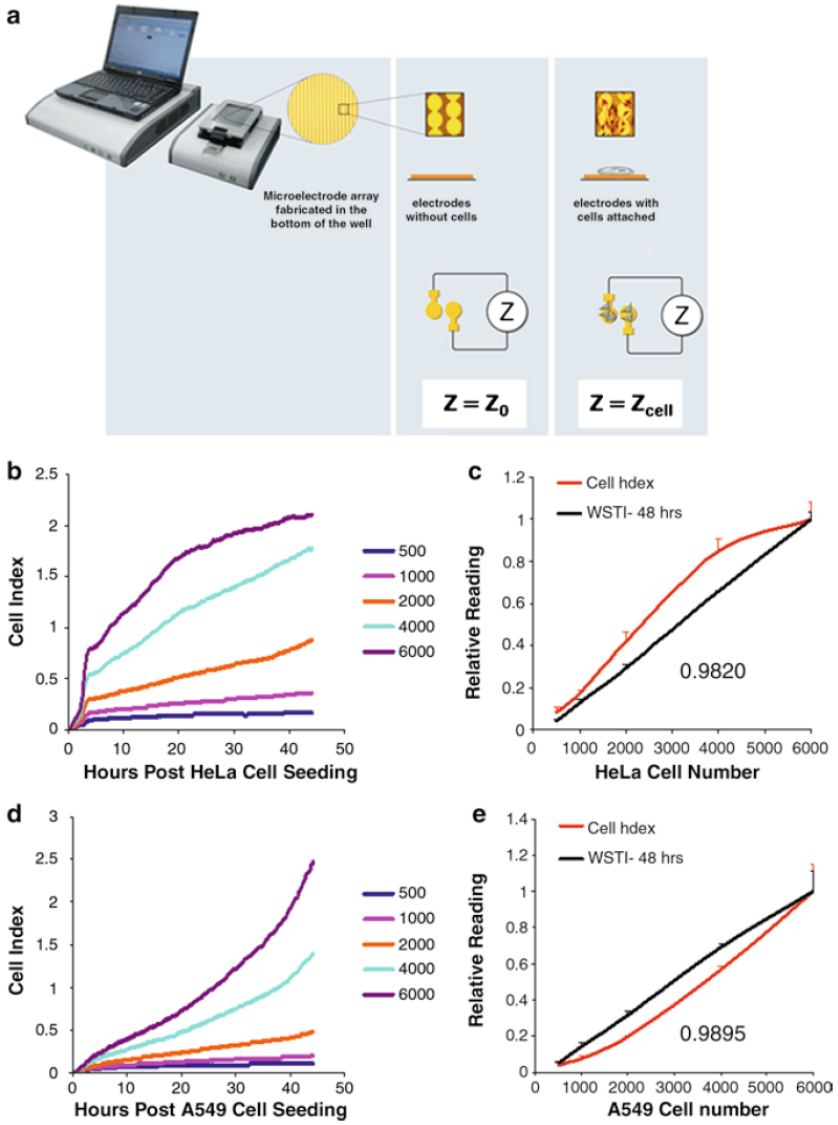

Fig. 1. xCELLigence in real-time monitoring of cell proliferation. (**a**) RTCA-SP instrument is shown, consisting of control unit, station, E-Plate, and display unit. Interdigitated gold electrodes at the bottom of the E-Plate are shown with or without cells. Background impedance is determined for medium alone (Z_0), then after the cells attach and proliferate on the electrodes (Z_{cell}). (**b**–**e**) Different numbers of HeLa (**b**) and A549 (**d**) cells were seeded in the E-Plate and the cell index was continuously monitored for 45 h. At the end of study, WST-1 was added to the medium and absorbance determined (**c** and **e**). Both cell index and WST-1 readings at the end of study were normalized against the value obtained at the highest seeding density. The average of triplicate samples is plotted with error bars indicating standard deviation. The Pearson correlation coefficient between WST-1 and cell index readings is also indicated.

Ning Ke, Xiaobo Wang, Xiao Xu, and Yama A. Abassi. The xCELLigence System for Real-Time and Label-Free Monitoring of Cell Viability. Chapter 6. Martin J. Stoddart (ed.), *Mammalian Cell Viability: Methods and Protocols*, Methods in Molecular Biology, vol. 740,
DOI 10.1007/978-1-61779-108-6_5,

Label-free technologies

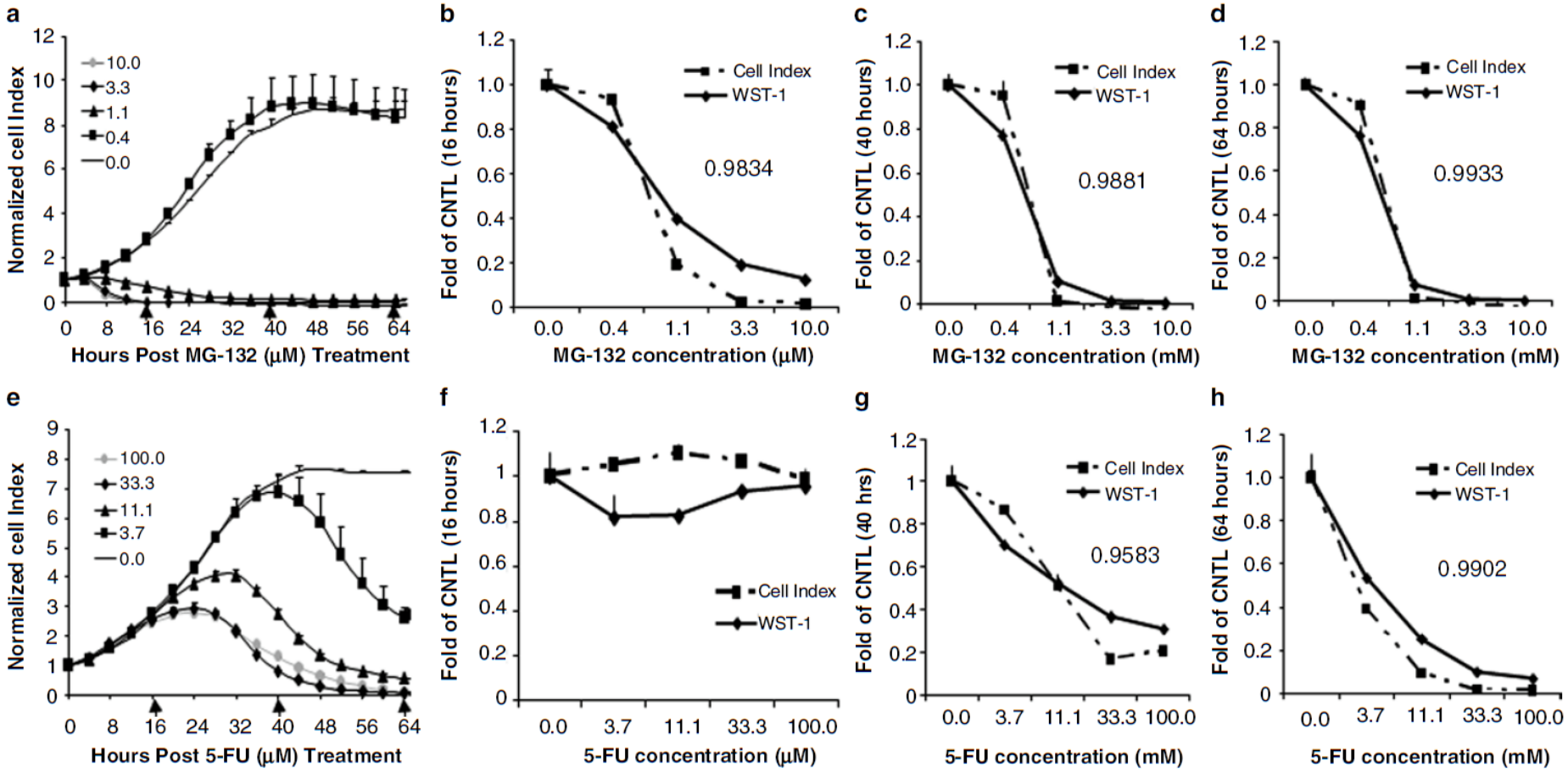

Fig. 2. xCELLigence system allows real-time monitoring of cell viability. HeLa cells were treated with various concentrations of the proteasome inhibitor MG-132 (**a**–**d**) and the DNA-damaging agent 5-FU (**e**–**h**). Cell index was monitored continuously for 64 h and the normalized cell index was derived by dividing the cell index value at each time point by the cell index at time of compound additions and plotted (**a** and **e**). No compound (no cpd) was used as control. Parallel experiments were also set up in E-plates for the WST-1 assay, which was performed at 16, 40, and 64 h post compound addition (**b**–**d** and **f**–**h**). Normalized cell index and WST-1 readings against control samples (untreated) were shown at the respective time points (16 h, **b** and **f**; 40 h, **c** and **g**; 64 h, **d** and **h**). The average of triplicate samples was shown and the error bars indicate standard deviation. The Pearson correlation coefficient was also shown for each case where cytotoxicity was observed.

Label-free technologies

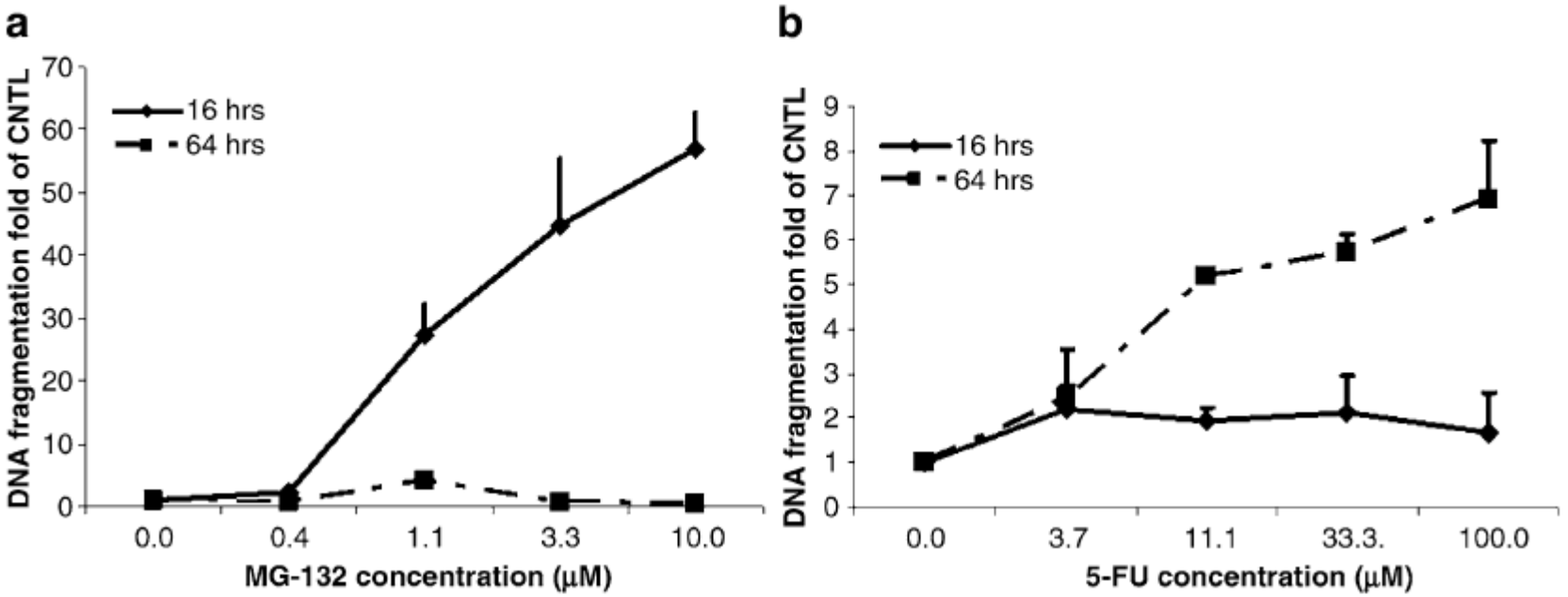

Fig. 3. Cell index changes and apoptosis. HeLa cells were treated with various concentrations of the proteasome inhibitor MG-132 (**a**) and the DNA-damaging agent 5-FU (**b**) and the cell index was monitored continuously for 64 h as in Fig. 2. A parallel experiment was set up in an E-plate and samples were harvested at 16 (*solid line*) and 64 h (*dotted line*) post compound addition for apoptosis assays (**a** and **b**). Apoptosis was determined using Cell Death Detection Elisa-Plus kit, and was expressed as the ratio of DNA fragmentation signal to that of the untreated samples. While the proteasome inhibitor MG-132 exerts its effect on cell index as early as 4 h post treatment, with maximal effect at 10 h post treatment, 5-FU effect is much slower, with onset at ~16 h post treatment, and the maximal effect observed after 48 h. Correspondingly, apoptosis induction is observed at 16 h for MG-132, and at 64 h for 5-FU. Importantly, no apoptosis induction is observed for MG-132 at 64 h, confirming the transient nature of apoptosis and indicating the importance of continuous monitoring of cell viability in selecting optimal time points for end-point assays.

Microcalorimetry

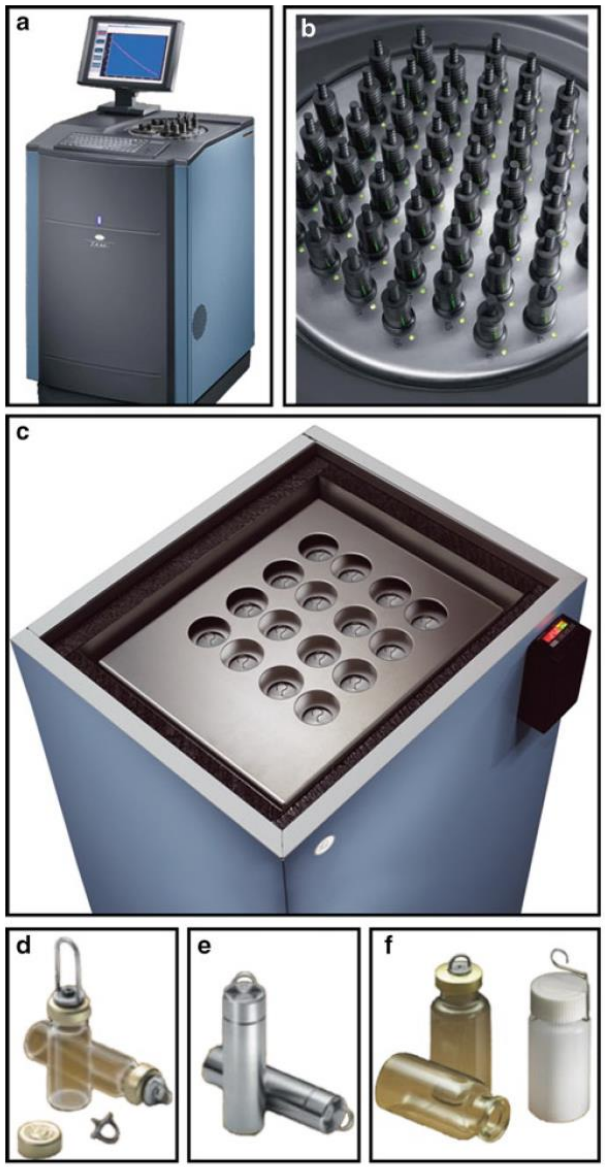

Fig. 1. Examples of commercially available isothermal microcalorimetry (IMC) instruments with multiple microcalorimeters or calorimeters, and ampoules for closed ampoule studies. All instruments and ampoules: Waters/TA, New Castle DE USA (images: courtesy of Waters/TA) (**a**) General view of a TAM III® equipped with various microcalorimeters. (**b**) Top view of a TAM 48® with its array of 48 microcalorimeters (i.e. 48 individual measuring channels). (**c**) Top view of a TAM Air® showing its eight individual differential calorimeters. (**d**) 3- and 4-ml disposable glass ampoules. (**e**) 4-ml stainless steel ampoules. (**f**) 20-ml disposable glass and polyethylene ampoules.

Olivier Braissant and Alma U. Daniels. Closed Ampoule Isothermal Microcalorimetry for Continuous Real-Time Detection and Evaluation of Cultured Mammalian Cell Activity and Responses. Chapter 20
Martin J. Stoddart (ed.), *Mammalian Cell Viability: Methods and Protocols*, Methods in Molecular Biology, vol. 740,
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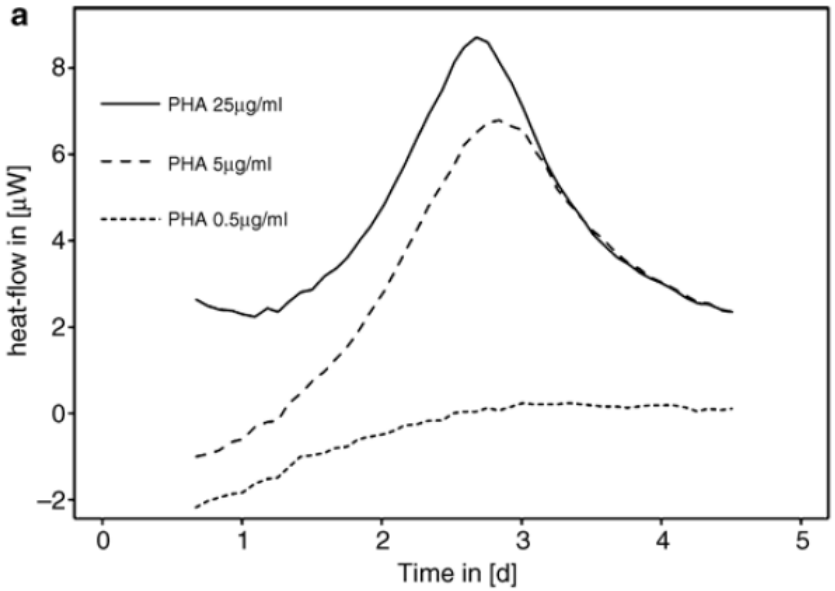
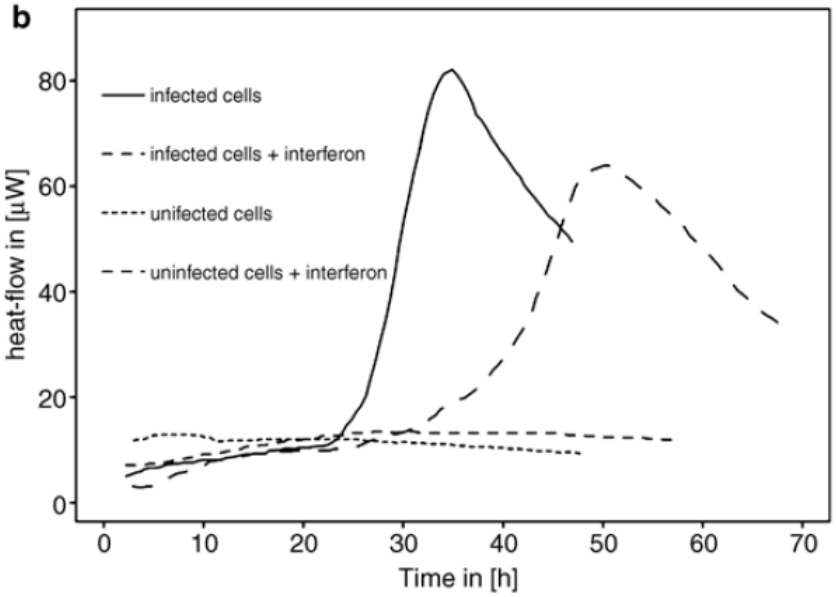
Microcalorimetry





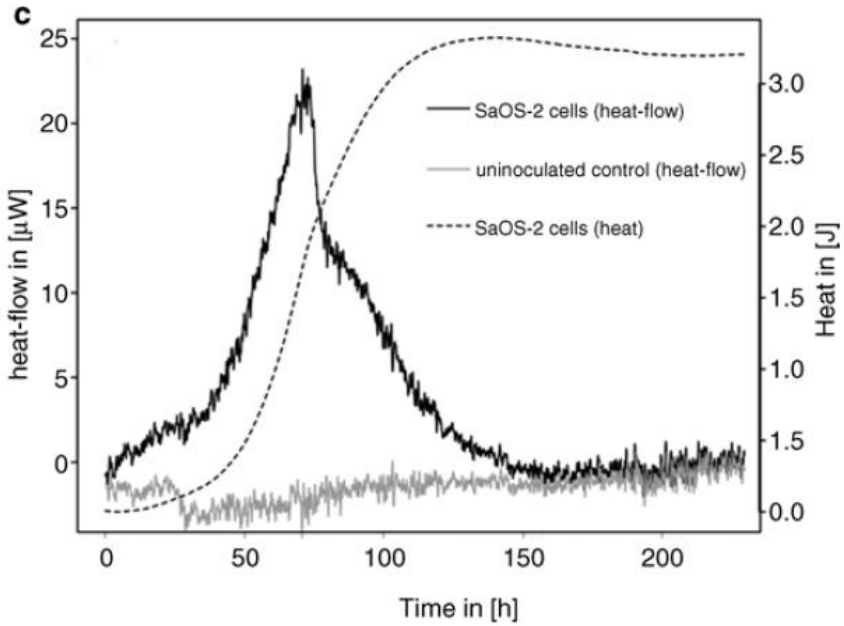


Fig. 4. Examples of heat flow data obtained following protocols.
(**a**) Monitoring lymphocyte activity showing heat flow from lymphocytes (initial amount 2.5×10^5 cells) over a period of days when stimulated with various amounts of phytohemagglutinin (PHA).

(**b**) Monitoring virus infection in mammalian cells, showing heat flow from BHK-21 cells at 37°C.
(a) FMDV infected cells (*plain line*), (b) infected cells with 1.0 mg/ml interferon (*dashed line*), (c) uninfected cells with 1.0 mg/ml interferon (*dashed line*), and (d) uninfected cells without interferon (*dashed line*). (**c**) Monitoring growth of SaOS-2 (human sarcoma osteogenic) cells (authors' data). *Plain dark line*: cells in culture, Plain grey line: inoculated control, *dashed line*: accumulated heat (J) vs. time, base on heat flow curve obtained during growth.
Accumulated heat vs. time is analogous to conventional growth curves showing, e.g. the accumulated number of cells.