MEDICAL BIOLOGY PRACTICALS. CYTOLOGY. Practical 4. Differentiation between living and dead eukaryotic cells by staining.

Chapter · December 2017

CITATIONS
0

READS
3,600

3 authors, including:

Boris M. Sharga
Uzhhorod National University
60 PUBLICATIONS **85** CITATIONS

SEE PROFILE

Volodymir Feketa
Uzhhorod National University
57 PUBLICATIONS **4** CITATIONS

SEE PROFILE

Uzhhorod National University
Medical Faculty №2
Department of Fundamental Medical Disciplines

MEDICAL BIOLOGY PRACTICALS. CYTOLOGY.

Practical 4. Differentiation between living and dead eukaryotic cells by staining.

Compiled by Boris M. Sharga, Diana B. Pylypiv, Volodymir P. Feketa

Theoretical background. There are several stains for differentiation of dead and living eukaryotic cells. Skeletal formulae are shown for some of them in Fig.1-6.

Methylene blue (MB) stain is used to highlight parts of plant, animal and blood tissue, bacteria and fungi. **MB** stains acidic cell parts (like nuclear DNA or RNA) blue and is a good counterstain with eosin Y. It can be substituted for Janus Green B stain or Carmine stain. It is also used to differentiate between dead and alive cells of eukaryotes [1, 12]. Dead eukaryotic cells are stained quickly in blue. Yeasts living cells contain an enzyme that discolors (reduces) **MB** and they appeared unstained. However, dead cells are unable to reduce the oxidized **MB** and the cells are stained blue. **MB** can interfere with the yeast respiration as it picks up hydrogen ions made during the process.

MB stains negatively charged molecules in the cell, including DNA and RNA. The dye is toxic, as it contains aromatic rings, one of which is heterocyclic, methyl groups and sold as chloride (Fig.1). So it is toxic, when ingested and it causes irritation when in contact with the skin and eyes.

Erythrosine (disodium salt of 2, 4, 5, 7- tetraiodofluorescein, Fig. 2) is an organoiodine compound, a derivative of fluorone. It is cherry-pink synthetic, primarily used for food coloring (E127, Red No 3) [4, 22]. It is the disodium salt of 2,4,5,7-tetraiodofluorescein. It has absorbance max at 530 nm in water and it is photodegradable. Gives pink cherry color to the dead, but not to alive eukaryotic cells. It is usable as dental plaque disclosing agent also [30]. High doses of the substance have been found to cause thyroid cancer in rats [11]. Its use is restricted in some countries, because of ability to cause health problems [4].

Trypan blue (TB) or Diamine blue, Niagara blue and Trypan red were first synthesized by P.Ehrlich in 1904. **TB** is a dye of cotton textiles industry [8]. In biomedical sciences, it is used to stain fungi, protists and to selectively stain dead tissues or separate cells in blue. Live cells or tissues with intact plasma membranes (PM) are not stained. Since PM are very selective in the compounds that pass through them, in a viable cell **TB** is not accumulated. However, it penetrates the PM of dead cells. Since live cells are excluded from being stained, the method is often called as a *dye exclusion method*. **TB** colors the dead cells in blue, but living cells with intact membranes remained uncolored. **TB** is so-called because it can kill trypanosomes, the sleeping sickness agents. An analog of **TB**, the suramin is used against trypanosomiasis now.

As we see from skeletal formula (Fig. 3), the **TB** contains toxic aromatic rings and some toxic side groups. This dye may be a cause of certain birth defects such as *encephalocele (cranium bifidum)*, a defect of neural tube development in fetus characterized by sac-like protrusions of the brain and its membranes through pathological openings in the skull [19].

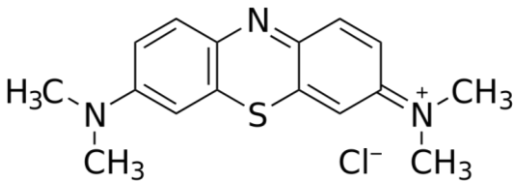

Fig. 1. Methylene blue. By Calvero [3].

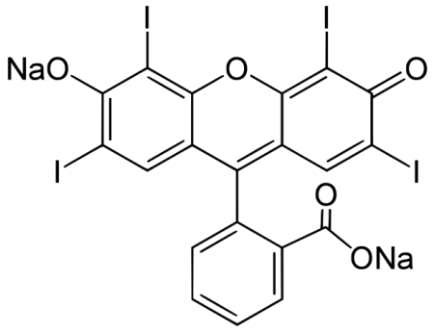

Fig. 2. Erythrosine [9].

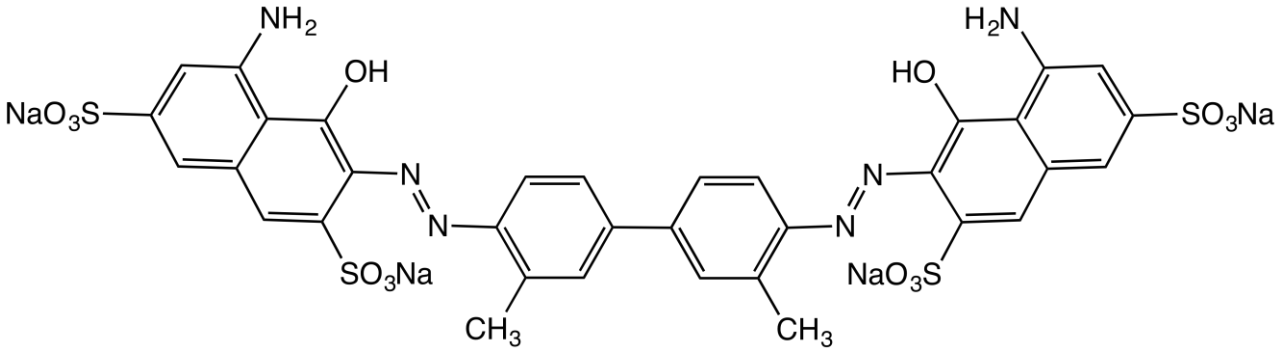

Fig. 3. Trypan blue. By Smokefoot [27].

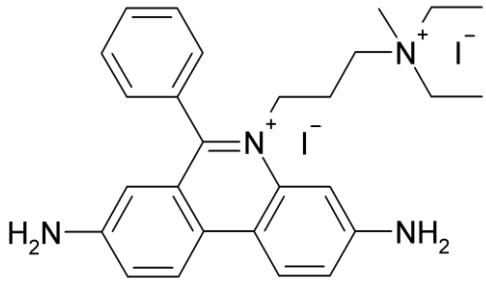

Fig 4. Propidium iodide. By K.Hoffmeier [13].

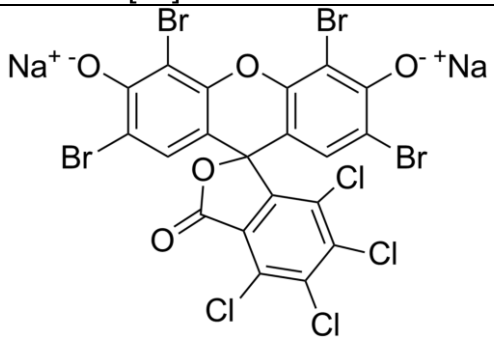

Fig. 5. Phloxine B. By Edgar [5].

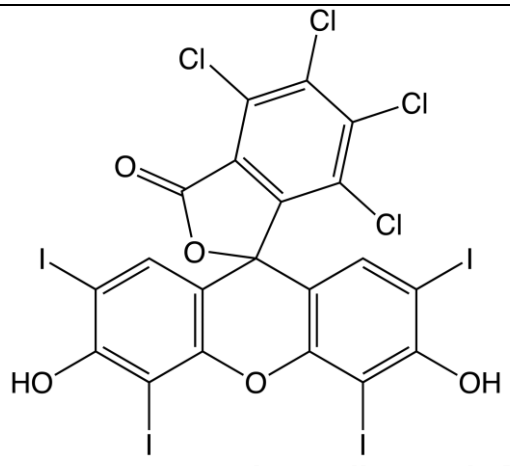

Fig. 6. Rose Bengal. By Elbreapoly [6].

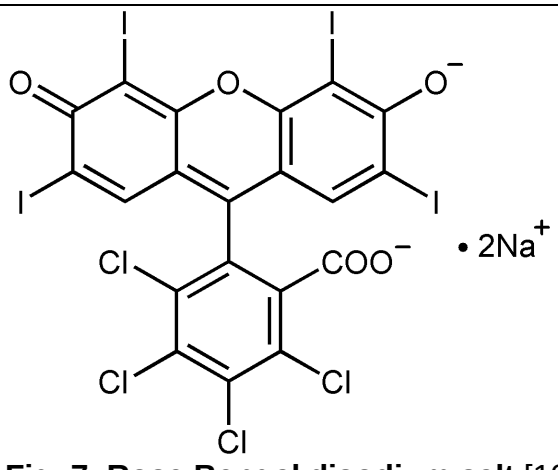

Fig. 7. Rose Bengal disodium salt [10].

Propidium iodide (**PI**) is an intercalating agent and a fluorescent molecule that can be used to stain cells. It is positively charged in solutions (Fig. 4) and binds to negatively charged NA. It fluoresces in red at 488 nm. Used as a DNA stain for both: in flow cytometry to evaluate cell viability or DNA content in cell cycle studies and in microscopy to visualize the nucleus and other DNA-containing organelles. **PI** also binds to RNA. The treatment with nucleases (RNA-ase or DNA-ase) followed by **PI** staining allows to differentiate between RNA and DNA containing material [28]. It can be used to differentiate necrotic, apoptotic and normal cells [16]. It penetrates plasma membrane in dead cells only, providing red fluorescence of nuclear regions of dead cells. Alive cells are not stained.

Phloxine B (PB) is used as coloring agent for drug, cosmetics [7] and food [14]. It has 4 bromine and 4 chlorine atoms in its structure. The absorption and emission maximums of the compound are around 540 nm and 564 nm, respectively. **PB** is an antimicrobial [25], viability dye and stain in hematoxylin-phloxine-saffron method to color the cytoplasm or connective tissue in red [2].

PB is negatively ionizes in water and binds to positively charged cell components. When **PB** is light subjected, debromination occured, free radicals and singlet oxygen are formed. These damage bacteria irreversibly, causing cell growth arrest and death [26]. Gram-negative bacteria are **PB**-resistant due to the outer cell membrane, a polysaccharide-coated lipid bilayer, a permeability barrier that prevents efficient **PB** uptake.

In cell count, the number of fluorescent (i.e. dead) cells observed can be compared to the total number of cells to give a measure of mortality [20].

Rose Bengal (RB) is 4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein. **RB** has been proved as antimicrobial sealing and collagen fibers linking agent for wounds [18, 21, 23], which fasters the healing process [15]. A **RB** forms are also being in clinical trials as remedy for melanoma, eczema, psoriasis [24] and breast cancer [17].

Its sodium salt (Fig. 6) is used to stain damaged cells of conjunctiva or cornea of the eye *in pink*. The stain is also used as protoplasm stain in microscopy to discriminate between alive or dead cells, particularly in protists *Foraminifera* [29].

Experiment 1. Differentiation between living and dead cells of yeast

Materials. Glass microscope slides and cover slips, light microscope, pipette, sterile tubes, sterile tap water, paper towel, laboratory gloves, bakery yeasts (*Saccaromyces cerevisiae*), methylene blue solution (1%).

Method

1. Pour 3-4 ml of sterile tap water in a tube.
2. Dissolve the approximately 5 mg yeasts in it to produce suspension about 10^5-10^8 cells/ml (the frame of the window should be visible through it).
3. Make drop preparation of it on glass with use of cover slip.
4. Using the smallest magnification objective, check if suspension is not too concentrated for easy calculation of the cells. If so, make ten-fold dilutions of the suspension.
5. Methylene blue is toxic. **Wear gloves!** Add a drop of methylene blue solution from sides of the cover slip.

6. Allow several minutes for cells to stain. Remove the excess of liquid by touching side of the coverslip with edge of paper towel.
7. Recognize the round and oval dead dark-blue or living non-stained cells as it is pictured in Fig. 8.

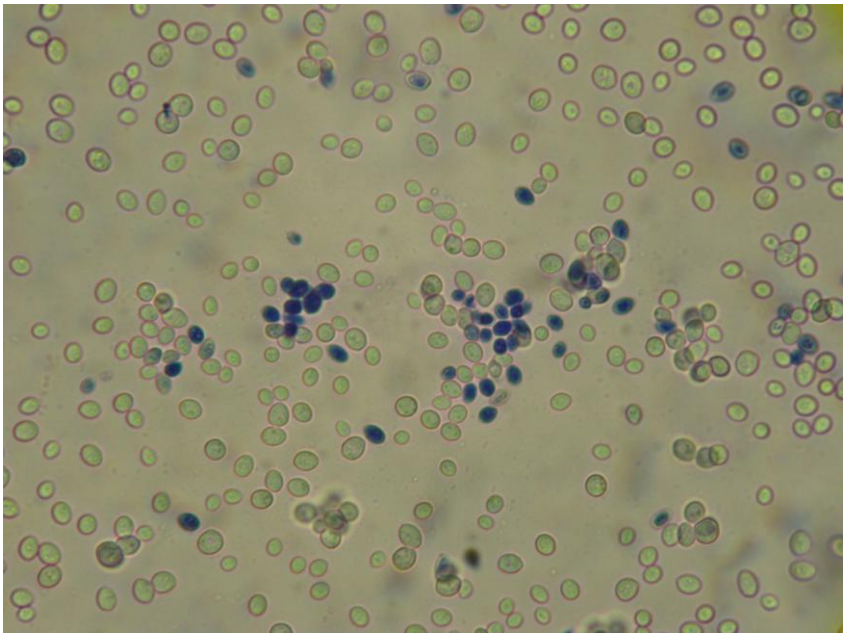

Fig. 8. Methylene blue stained *Saccharomyces cerevisiae* cells. The nuclei of just died cells turned blue, the cytoplasm is less stained yet. Later whole dead cells develop blue color. By B.M.Sharga.
Magnification 600 ×.

8. Calculate the average percentage of dead cells by dividing their average number onto total average number of cells into several fields of view and multiplying the result by 100%:
   Dead cells % = (dead cells number mean/total number of cells mean) × 100%
9. Make conclusion from the observations about viability of cells in bakery yeasts and provide an explanation of staining results. Draw a picture of dead and alive cells in your note.

Experiment 2. Staining of squamous epitelium cells from human throat lining

Materials. Glass microscope slides and cover slips, sterile individually packed cotton swabs, paper towel, laboratory gloves, methylene blue solution (1%).

Method

1. Scrape gently the inside of your mouth cheek by sterile cotton swab.
2. Smear the cotton swab on the microscope slide.
3. Methylene blue is toxic. **Wear gloves!** Pipet a drop of methylene blue solution and position a coverslip over it. Remove the solution excess by touching side of the coverslip with edge of paper towel.
4. Place the slide on the microscope stage, position objective 9× or 10× and find the best field of view with the cells. Then observe cells from outer layer of squamous epithelium of your cheeck at higher magnification using the objective 40×. The small blue dots and rods on their surface are cocci and rod-shape bacteria from

residential flora of throat or transient microbes from food. You might see the cells as in Fig. 9.

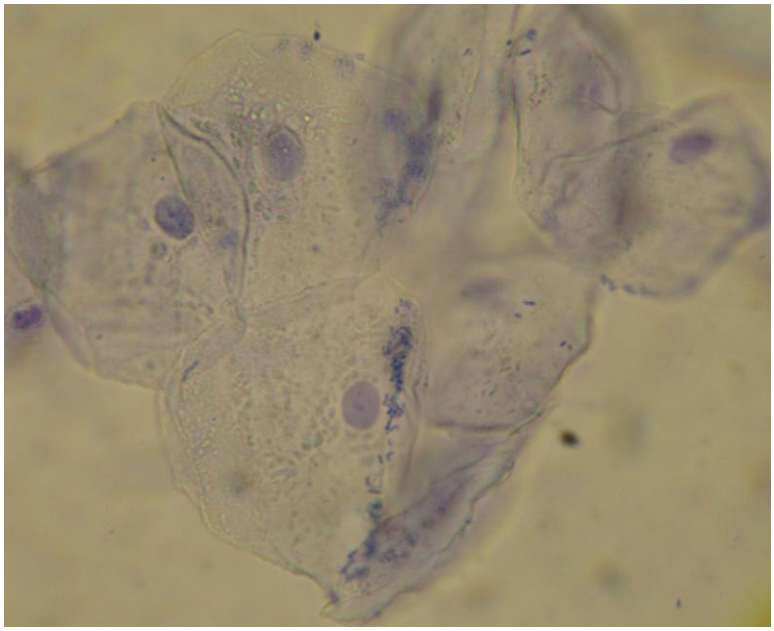

Fig. 9. Squamous epitelium cells from human throat lining. Note bacteria on the surface of the cells. By D.B.Pylypiv.
Magnification 600 ×.

5. Buccal cells continually secrete mucus and together with saliva from salivary glands maintain a moist environment in the mouth for digestive enzymes to thrive. Recently, researchers have discovered that the cheek cell can serve to measure a person's likelihood of having hypertension.

What can you tell about viability of buccal cells pictured on Fig. 9?

Literature:

1. Біланич М.М., Шарга Б.М., Ніколайчук В.І. Метод диференціації мертвих і живих клітин поверхневих тканин і створення постійних препаратів // Наук. Вісн. Ужгродського ун-ту. - сер. “Біологія”.- 2008.- Вип. 22.- с. 19-20.

2. Borgerink, Hermina. "HPS stain". Narkive Mailing List Archive. 18 April 2016.

3. Calvero. https://commons.wikimedia.org/wiki/ File:Methyleneblue.svg)

4. Color Additive Status List. *FDA. Dec. 2015*. Retrieved May 16, 2016.

5. Edgar. https://upload.wikimedia.org/wikipedia/commons/ b/b1/Phloxine.png

6. Elbreapoly. https://en.wikipedia.org/wiki/Rose_bengal#/media/File:Rosebengalskeletal.png

7. Food and Drug Administration. The Code of Federal Regulations of the United States of America, Title 21, Part 74.1328. US Government Printing Office.- 2001.-p. 296.

8. Gurr E. Synthetic Dyes in Biology, Medicine And Chemistry.- Elsevier.- 2012 - p. 197- 198.

9. https://upload.wikimedia.org/wikipedia/commons/9/91/Erythrosine.svg

10. https://upload.wikimedia.org/wikipedia/commons/c/c7/Rose_bengal_sodium_salt_structure.png

11. Jennings A.S., Schwartz S. L., Balter N. J., Gardner D., Witorsch R. J. Effects of oral erythrosine (2',4',5',7'-tetraiodofluorescein) on the pituitary-thyroid axis in rats // Toxicology and Applied Pharmacology. -1990.- 103, Vol. 3.- p.549–56.

12. Jörgensen A. Micro-organisms and fermentation.- Charles Griffin and Company, London.- 1948- p.275.

13. K.Hoffmeier. https://commons.wikimedia.org/wiki/File:Propidium_iodide.png

14. Kamikura M. "Thin Layer Chromatography of Synthetic Dyes (X)". Food Hygiene and Safety Science (Shokuhin Eiseigaku Zasshi).- 1970.- Vol.**11.-**№4.- p. 242–248.
15. Laser Show in the Surgical Suite, Technology Review, 02.11.2009
16. Lecoeur H. Nuclear apoptosis detection by flow cytometry: influence of endogenous endonucleases//Exp. Cell Res. 2002.- Vol.277 №1. - p. 1–14.
17. Metastatic Melanoma PV-10 Trial Results Encouraging Says Drug Company, Medical News Today, 05.09.2009 http://www.medicalnewstoday.com/articles/153024.php
18. Mulroy L., Kim J., Wu I., Scharper P., Melki S.A., Azar D.A., Redmond R.W., Kochevar I.E. Photochemical keratodesmos for repair of lamellar corneal incisions// Invest. Ophthalmol. Vis. Sci. 2000.- Vol.**41,** №11. - p. 3335–3340.
19. NINDS Encephaloceles Information Page, NINDS, http://www.ninds.nih.gov/disorders/encephaloceles/encephaloceles.htm
20. Noda T. Viability assays to monitor yeast autophagy// Meth. Enz. 2008.- Vol. **451**: p. 27–31.
21. O'Neill A.C., Winograd J.M, Zeballos J.M., Johnson T.S., Randolph M.A., Bujold K.E., Kochevar I.E., Redmond R.W. Microvascular anastomosis using a photochemical tissue bonding technique // Lasers in Surgery and Medicine. 2007.- Vol. **39,** №9. – p.716–722.
22. Phyllis A. Lyday Iodine and Iodine Compounds. *In:* Ullmann's Encyclopedia of Industrial Chemistry, Wiley-VCH, Weinheim, 2005
23. Proano C.E.; Mulroy L.; Erika Jones E.; Azar D.A.; Redmond R.W.; Kochevar I.E. Characterization of paracellular penetration routes // Invest. Ophthalmol. Vis. Sci. 2004.- Vol. **38.**- p. 2177–2181.
24. Provectus Biopharmaceuticals Reports Data On PV-10 in Combination Therapy and T Cell Mediated Immunity.- American Association for Cancer Research (AACR) Annual Meeting 2016.- http://www.businesswire.com/news/home/20160422005164/en/Provectus-Biopharmaceuticals-Reports-Data-PV-10-Combination-Therapy
25. Rasooly A.; Weisz A. *In vitro* Antibacterial Activities of Phloxine B and Other Halogenated Fluoresceins against Methicillin-Resistant *Staphylococcus aureus*// Antim. Agents and Chemother.- 2002.-Vol.46 №11.- p. 3650–3653.
26. Rasooly, Reuven. Expanding the bactericidal action of the food color additive phloxine B to gram-negative bacteria// FEMS Immunol. and Med. Microbiol. 2005.- Vol.**45**.- p.239–244.
27. Smokefoot. https://commons.wikimedia.org/wiki/File:TrypanBlueSalt.png
28. Suzuki T., Fujikura K., Higashiyama T., Takata K. DNA staining for fluorescence and laser confocal microscopy// J. Histochem. Cytochem.-1997.- Vol.**45** №1.-p. 49–53.
29. Walton W. Techniques for recognition of living foraminifera //Contrib. Cushman Found. Foraminiferal Res.- 1952.- Vol.3.- p.56–60.
30. Wood S., Metcalf D., Devine D., Robinson C. Erythrosine is a potential photosensitizer for the photodynamic therapy of oral plaque biofilms//J. of Antimic. Chemother.- 2006.-Vol. 57, №4.- p. 680-684.