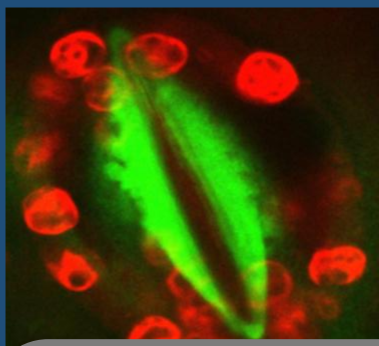
Center of Excellence at Orbeli Institute of Physiology

Funded by the European Union

CHAN ZUCKERBERG INITIATIVE

TEXTBOOK OF PROTOCOLS

CONFOCAL MICROSCOPY



CELL STAINING

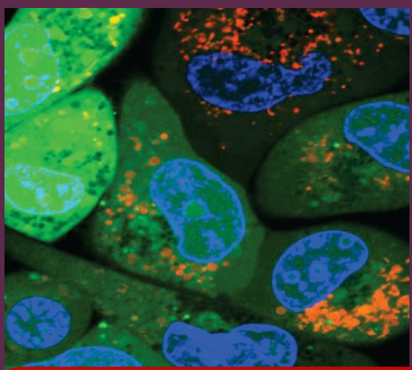
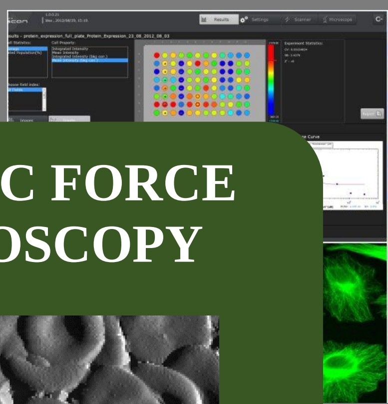
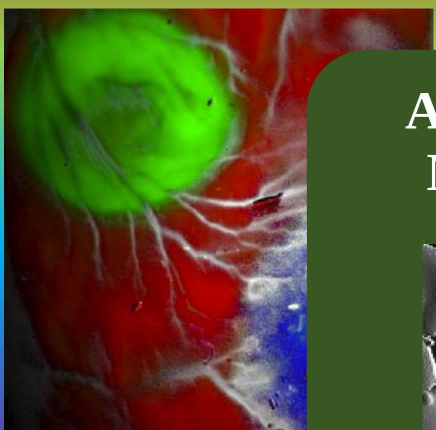


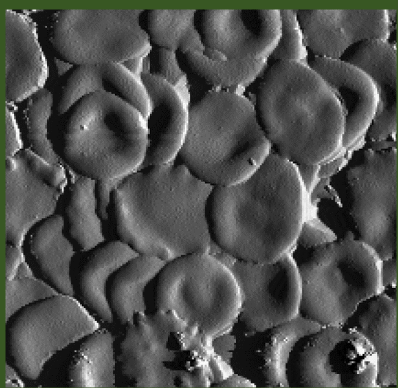
IMAGE PROCESSING



HYPERSPECTRAL IMAGING



ATOMIC FORCE MICROSCOPY



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SOURCES & ACKNOWLEDGEMENTS

This manual on key biomedical imaging techniques and protocols was compiled based on a series of bioimaging courses conducted at the Orbeli Institute of Physiology in Yerevan, Armenia. These activities, initially launched with the support of the Chan Zuckerberg Initiative (CZI), were later developed and expanded through the ERA Chair project funded by the European Union.

Multiple online sources were used in compiling these educational materials, all of which were made freely available to numerous students. These sources include, but are not limited to, the following:

- Zeidan, C. A. & Yehiam, L. *Fiji / ImageJ Hands-On Training.*
- Young, K. & Morrison, H. *Quantifying Microglia Morphology from Photomicrographs of Immunohistochemistry Prepared Tissue Using ImageJ.* JoVE 57648 (2018) doi:10.3791/57648.
- Wang, Y. W., Reder, N. P., Kang, S., Glaser, A. K. & Liu, J. T. C. *Multiplexed Optical Imaging of Tumor-Directed Nanoparticles: A Review of Imaging Systems and Approaches. Nanotheranostics* 1, 369–388 (2017).
- Sanka, I., Bartkova, S., Pata, P., Smolander, O.-P. & Scheler, O. *Investigation of Different Free Image Analysis Software for High-Throughput Droplet Detection. ACS Omega* 6, 22625–22634 (2021).
- McMillan, D. P. *FIJI/ImageJ for Beginners: Fundamentals of Image Quantification.*
- Canterero, V. L. *Image Processing and Object Segmentation using Fiji/ImageJ.*
- Bankhead, P. *Analyzing fluorescence microscopy images with ImageJ.*
- Chen, Y., Cai, J., Murphy, T. J. & Jones, D. P. *Overexpressed Human Mitochondrial Thioredoxin Confers Resistance to Oxidant-induced Apoptosis in Human Osteosarcoma Cells. Journal of Biological Chemistry* 277, 33242–33248 (2002).
- Vermaas, W. F. J. et al. *In vivo hyperspectral confocal fluorescence imaging to determine pigment localization and distribution in cyanobacterial cells. Proc. Natl. Acad. Sci. U.S.A.* 105, 4050–4055 (2008).
- Ortega, S. et al. *Hyperspectral Imaging for the Detection of Glioblastoma Tumor Cells in H&E Slides Using Convolutional Neural Networks. Sensors* 20, 1911 (2020).
- Ishikawa, M. et al. *Detection of pancreatic tumor cell nuclei via a hyperspectral analysis of pathological slides based on stain spectra. Biomed. Opt. Express* 10, 4568 (2019).
- Hadoux, X. et al. *Non-invasive in vivo hyperspectral imaging of the retina for potential biomarker use in Alzheimer's disease. Nat Commun* 10, 4227 (2019).

USEFUL LINKS:

- *https://ibidi.com/content/216-confocal-microscopy*
- *https://www.thermofisher.com/order/fluorescence-spectraviewer#!/*
- *https://www.mdpi.com/2072-6694/11/6/756*
- *https://www.teledynevisionsolutions.com/learn/learning-center/scientific-imaging/filters/*
- *https://midopt.com/filters/*
- *https://www.leica-microsystems.com/science-lab/*
- *https://arxiv.org/abs/1708.03568*
- *https://link.springer.com/chapter/10.1007/978-981-15-9472-4_64*
- *https://micro.magnet.fsu.edu/primer/techniques/dic/dicphasecomparison.html*
- *https://www.brainkart.com/article/Phase-Contrast-Microscope_40956/*
- *https://www.bcm.edu/research/faculty-labs/ross-poche-lab*
- *https://www.zeiss.com/microscopy/en/products/light-microscopes/confocal-microscopes.html*
- *https://academic.oup.com/genetics/article/211/1/15/5931125*
- *https://www.researchgate.net/publication/336107044_Investigating_Ions'_Effects_on_the_Fluorescent_Protein_Dendra2*
- *https://ieeexplore.ieee.org/document/8039195*
- *https://warwick.ac.uk/fac/sci/lifesci/research/facilities/imaging/sp5booking/imaging-pinhole/*
- *https://pubmed.ncbi.nlm.nih.gov/24974025/*
- *https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/confocal/resolutionintro/*
- *https://link.springer.com/article/10.1007/s00105-015-3632-y*

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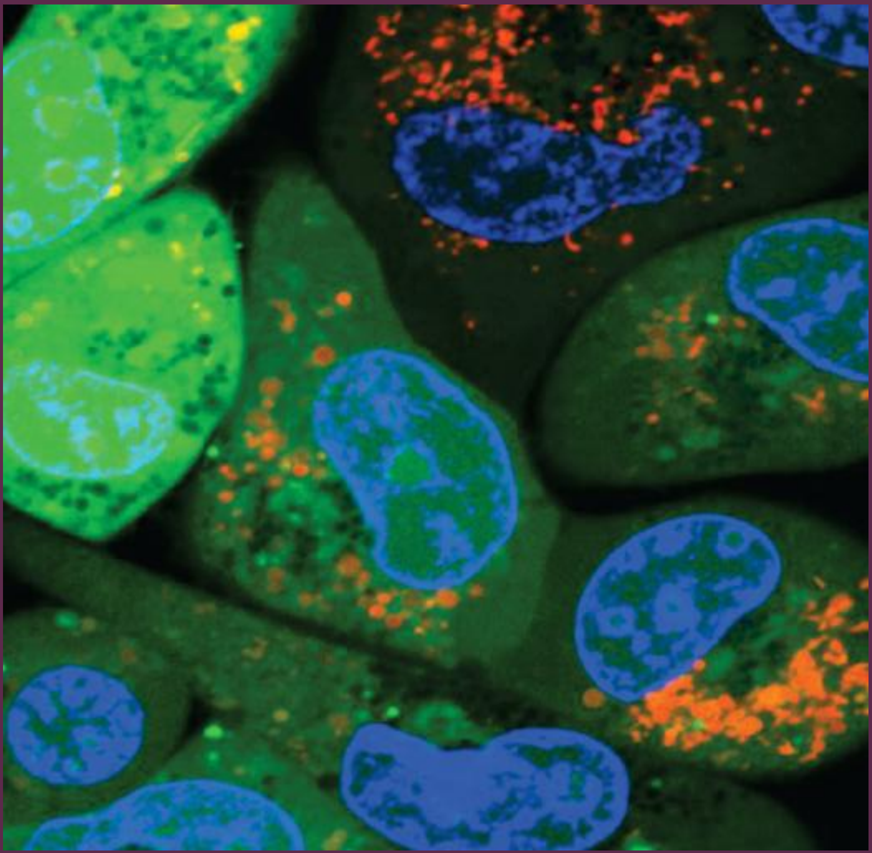
CELL STAINING



INTRODUCTION

The Cell Staining course provides a comprehensive overview of the principles and practical applications of fluorescence-based cell staining methods. It focuses on the fundamental concepts of fluorescence, the selection and utilization of fluorescent dyes for visualizing organelles, immunostaining techniques, and live-cell imaging approaches. Throughout the course, participants will engage in hands-on laboratory sessions, where they will practice cell staining techniques using fluorescent dyes and antibodies.

Cell staining is a fundamental technique used in biology and medical research to enhance the visibility of cells and their components under a microscope. Since most cells are transparent and colorless, it can be challenging to distinguish their various structures, such as the nucleus, mitochondria, and other organelles, without some form of contrast. Staining provides this contrast by applying dyes or stains that selectively bind to specific cellular components, allowing scientists to observe and study cells in greater detail.

The process of cell staining involves several steps, beginning with the preparation of the specimen. Cells or tissues are typically fixed to preserve their structure, which involves treating them with chemicals that "freeze" their cellular components in place. After fixation, the sample is stained with one or more dyes that are chosen based on the specific structures or molecules of interest.

There are many types of stains, each with unique properties and applications. Some stains, like Hematoxylin and Eosin (H&E), are used in routine histology to differentiate between different tissue types. Hematoxylin stains cell nuclei blue, while Eosin stains the cytoplasm and extracellular matrix pink, providing a clear contrast that is essential for examining tissue morphology.

Cell staining can be broadly categorized into simple staining, differential staining, and specialized staining. Simple staining involves the use of a single dye to color all cells or structures uniformly, making it useful for quickly observing the general shape and arrangement of cells. Differential staining, on the other hand, uses multiple stains to distinguish between different types of cells or between different structures within a single cell. For example, the Gram stain, a widely used differential stain, can differentiate between Gram-positive and Gram-negative bacteria based on the composition of their cell walls.

Specialized staining techniques, such as immunohistochemistry (IHC) and immunofluorescence, go a step further by using antibodies to target specific proteins within cells. These techniques are invaluable for identifying and locating specific molecules within complex tissues, making them crucial for research in cell biology, pathology, and molecular biology.

Cell staining is not only important for visualizing cells but also for diagnosing diseases. In clinical settings, stained tissue samples are routinely examined under a microscope to identify abnormal cell structures or to detect the presence of pathogens, such as bacteria and viruses. For example, Pap smears, which involve staining cells from the cervix, are used to screen for cervical cancer by identifying precancerous or cancerous cells.

Despite its advantages, cell staining has some limitations. The staining process can sometimes alter or damage the cells, leading to artifacts that may complicate interpretation. Additionally, some stains may not bind specifically, resulting in background staining that can obscure the structures of interest. However, with careful technique and appropriate controls, these issues can be minimized.

Ներածություն

Բջիջների ներկման դասընթացը տրամադրում է համապարփակ ներածություն՝ ֆլուորեսցենցային հիմքով բջջային ներկման մեթոդների տեսական հիմունքների և գործնական կիրառությունների վերաբերյալ: Այն կենտրոնանում է ֆլուորեսցենցիայի հիմնարար հասկացությունների, օրգանոիդների վիզուալիզացիայի համար ֆլուորեսցենտ ներկերի ընտրության և օգտագործման, իմունային ներկման տեխնիկայի և կենդանի բջիջների պատկերման մոտեցումների վրա: Դասընթացի ընթացքում մասնակիցները կմասնակցեն գործնական լաբորատոր պարապմունքների, որտեղ նրանք կկիրառեն բջիջների ներկման տեխնիկան՝ օգտագործելով ֆլուորեսցենտ ներկեր և հակամարմիններ:

Բջիջների ներկումը կենսաբանության և բժշկական հետազոտություններում օգտագործվող հիմնարար տեխնիկա է՝ բջիջների և դրանց բաղադրիչների տեսանելիությունը մանրադիտակի տակ բարձրացնելու համար: Քանի որ բջիջների մեծ մասը թափանցիկ և անգույն են, դրանց տարբեր կառուցվածքները, ինչպիսիք են կորիզը, միտոքոնդրիաները և այլ օրգանոիդները, տարբերակելը կարող է դժվար լինել առանց որևէ կոնտրաստի: Գունավորումը ապահովում է այս հակադրությունը՝ կիրառելով ներկանյութեր կամ ներկեր, որոնք ընտրողաբար կապվում են որոշակի բջջային բաղադրիչների հետ, թույլ տալով գիտնականներին ավելի մանրամասն դիտարկել և ուսումնասիրել բջիջները:

Բջիջների ներկման գործընթացը ներառում է մի քանի քայլ, որոնք սկսվում են նմուշի պատրաստումից: Բջիջները կամ հյուսվածքները սովորաբար ամրացվում են իրենց կառուցվածքը պահպանելու համար, ինչը ներառում է դրանց մշակում քիմիական նյութերով, որոնք «սառեցնում» են դրանց բջջային բաղադրիչները տեղում: Ֆիքսացիայից հետո նմուշը ներկվում է մեկ կամ մի քանի ներկանյութերով, որոնք ընտրվում են հետաքրքրության կոնկրետ կառուցվածքների կամ մոլեկուլների հիման վրա:

Գոյություն ունեն ներկերի բազմաթիվ տեսակներ, որոնցից յուրաքանչյուրն ունի յուրահատուկ հատկություններ և կիրառություն: Որոշ ներկեր, ինչպիսիք են հեմատոքսիլինը և էոզինը (H&E), օգտագործվում են հյուսվածաբանության մեջ՝ տարբեր հյուսվածքների տեսակները տարբերակելու համար: Հեմատոքսիլինը ներկում է բջջային կորիզները կապույտով, մինչդեռ էոզինը ներկում է ցիտոպլազման և արտաբջջային մատրիցի վարդագույնը՝ ապահովելով հստակ հակադրություն, որը կարևոր է հյուսվածքների ձևաբանությունը ուսումնասիրելու համար:

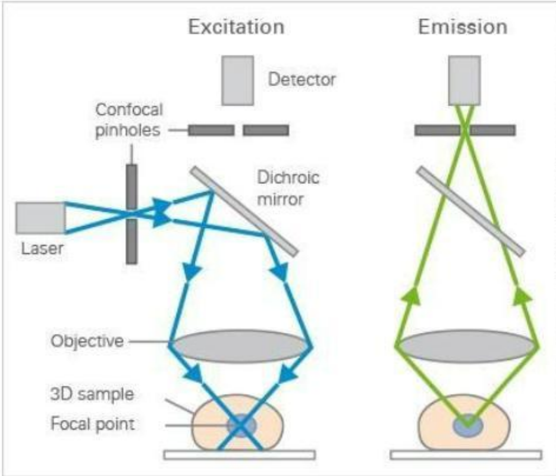
Բջջային ներկումը կարելի է լայնորեն դասակարգել պարզ ներկման, դիֆերենցիալ ներկման և մասնագիտացված ներկման: Պարզ ներկումը ներառում է մեկ ներկանյութի օգտագործում՝ բոլոր բջիջները կամ կառուցվածքները միատարր գունավորելու համար, ինչը այն օգտակար է դարձնում բջիջների ընդհանուր ձևը և դասավորությունը արագ դիտարկելու համար: Մյուս կողմից, դիֆերենցիալ ներկումը օգտագործում է բազմաթիվ ներկանյութեր՝ տարբեր տեսակի բջիջներ կամ մեկ բջջի ներսում տարբեր կառուցվածքներ տարբերակելու համար: Օրինակ, Գրամի ներկումը, որը լայնորեն օգտագործվող դիֆերենցիալ ներկ է, կարող է տարբերակել գրամ-դրական և գրամ-բացասական մանրէները՝ հիմնվելով դրանց բջջային պատերի կազմի վրա:

Մասնագիտացված ներկման տեխնիկաները, ինչպիսիք են իմունահյուսվածքաքիմիան (IHC) և իմունաֆլուորեսցենցիան, մեկ քայլ առաջ են գնում՝ օգտագործելով հակամարմիններ՝ բջիջների ներսում որոշակի սպիտակուցներ թիրախավորելու համար: Այս տեխնիկաները անգնահատելի են բարդ հյուսվածքներում որոշակի մոլեկուլներ նույնականացնելու և տեղորոշելու համար, ինչը դրանք կարևոր է դարձնում բջջային կենսաբանության, պաթոլոգիայի և մոլեկուլային կենսաբանության հետազոտությունների համար:

Բջիջների ներկումը կարևոր է ոչ միայն բջիջների վիզուալիզացիայի, այլև հիվանդությունների ախտորոշման համար: Կլինիկական պայմաններում ներկված հյուսվածքների նմուշները պարբերաբար հետազոտվում են մանրադիտակի տակ՝ բջջային աննորմալ կառուցվածքները նույնականացնելու կամ պաթոգենների, ինչպիսիք են մանրէները և վիրուսները, առկայությունը հայտնաբերելու համար: Օրինակ՝ ՊԱՊ թեստը, որը ներառում է արգանդի վզիկի բջիջների ներկում, օգտագործվում է արգանդի վզիկի քաղցկեղի սկրինինգի համար՝ նախաքաղցկեղային կամ քաղցկեղային բջիջները նույնականացնելով:

Չնայած իր առավելություններին, բջիջների ներկումն ունի որոշ սահմանափակումներ: Ներկման գործընթացը երբեմն կարող է փոփոխել կամ վնասել բջիջները, ինչը հանգեցնում է արտեֆակտների, որոնք կարող են բարդացնել մեկնաբանությունը: Բացի այդ, որոշ ներկումներ կարող են չկապվել հատուկ կերպով, ինչը հանգեցնում է ֆոնային ներկման, որը կարող է ծածկել հետաքրքրության ենթակա կառուցվածքները: Այնուամենայնիվ, զգույշ տեխնիկայի և համապատասխան վերահսկողության միջոցով այս խնդիրները կարող են նվազագույնի հասցվել:

PROTOCOLS AND VISUALS



Cell Staining, step-by-step training

Additional materials:

https://biotium.com/technology/cellular-stains/

https://www.abcam.com/secondary-antibodies/fluorescence-guide

https://www.labome.com/method/Live-Cell-Imaging.html

https://www.leica-microsystems.com/science-lab/introduction-to-live-cell-imaging/

Introduction

What is Cellular Staining?

Cell staining is a technique that can be used to better visualize cells and cell components under a microscope. By using different stains, one can stain certain cell components, such as a nucleus, a cell wall, or the entire cell. Most stains can be used on fixed, or non-living cells, while only some can be used on living cells; some stains can be used on either living or non-living cells.

Why Stain Cells?

The most basic reason that cells are stained is to enhance visualization of the cell or certain cellular components under a microscope. Cells may also be stained to highlight metabolic processes or to differentiate between live and dead cells in a sample. Cells may also be enumerated by staining cells to determine biomass in an environment of interest.

How Are Cells Stained and Slides Prepared?

Cell staining techniques and preparation depend on the type of stain and analysis used. One or more of the following procedures may be required to prepare a sample:

Fixation - serves to "fix" or preserve cell or tissue morphology through the preparation process. This process may involve several steps, but most fixation procedures involve adding a chemical fixative that creates chemical bonds between proteins to increase their rigidity. Common fixatives include formaldehyde, ethanol, methanol, and/or picric acid.

Permeabilization - treatment of cells, generally with a mild surfactant, which dissolves cell membranes in order to allow larger dye molecules to enter inside the cell.

Staining - application of stain to a sample to color cells, tissues, components, or metabolic processes. This process may involve immersing the sample (before or after fixation or mounting) in a dye solution and then rinsing and observing the sample under a microscope.

Mounting - involves attaching samples to a glass microscope slide for observation and analysis. Cells may either be grown directly to the slide or loose cells can be applied to a slide using a sterile technique. Thin sections (slices) of material such as tissue may also be applied to a microscope slide for observation.

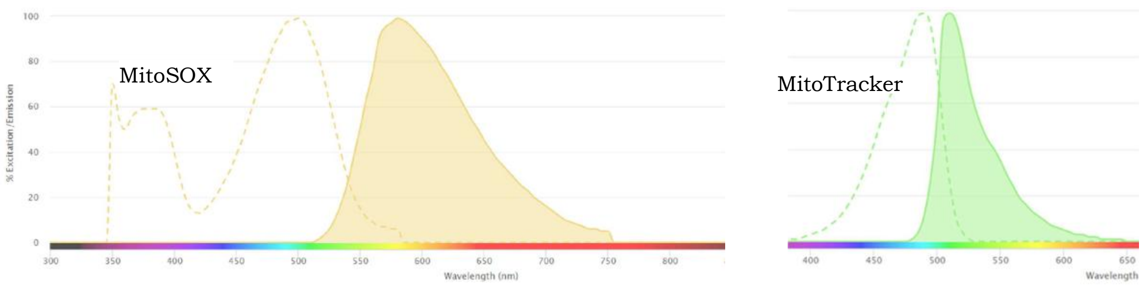
Part 1. Organelle staining

Eukaryotic cells contain subcellular structures responsible for carrying out essential functions for cell survival, including generating energy (ATP), synthesizing and trafficking proteins, and cell waste removal. Labeling key organelles, such as the cell membrane, nucleus, cytoplasm, mitochondria, lysosomes, endoplasmic reticulum (ER), Golgi apparatus, and cytoskeleton proteins (e.g., actin), can be used to monitor cell health, cell death, metabolic activity, autophagy, cell tracking, and cell migration.

Examples:

a. **Mitochondria**. Double-membrane-bound organelles, responsible for cellular respiration and the production of energy as adenosine triphosphate (ATP). Mitochondria are also involved in other tasks, such as cell signaling, cellular differentiation, and cell death—as well as maintaining control of the cell cycle and growth. Dependent on membrane potential, fluorescent live cell dyes for mitochondria can be used to analyze overall cell health and vitality, in addition to mitochondrial activity.

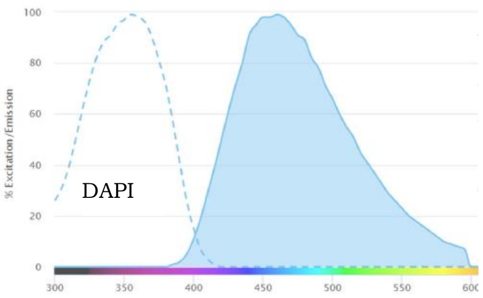
Name	Excitation/Emission (nm)
MitoSOX Red	510/580
MitoTracker Green	490/516

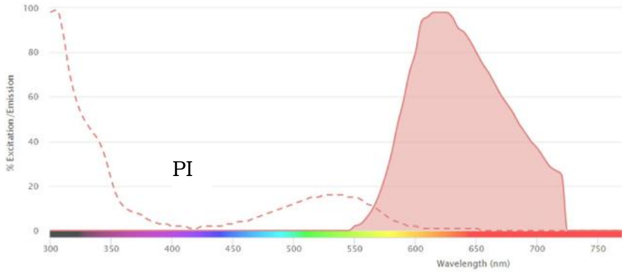


b. **Lysosome.** Membrane-bound organelles that act as the waste disposal system of the cell. As highly acidic (pH 4-5) organelles, lysosomes contain a variety of enzymes capable of breaking down various biomolecules including peptides, nucleic acids, carbohydrates, and lipids. In addition to biomolecule dissolution, lysosomes are also involved in autophagy and apoptosis. Depending on the internal acidic environment, live cell fluorescent dyes for lysosomes can be used to analyze lysosomal activity, autophagy events, vesicle trafficking, and overall cell health.

c. **Nucleus**. Containing nearly all of the cell's genetic material, the cell nucleus is organized in chromosomes, formed from the complexation of long linear DNA with proteins. Live cell nuclear stains can be used either for cell tracking, or as counterstains with other fluorescent dyes or cell reporters.

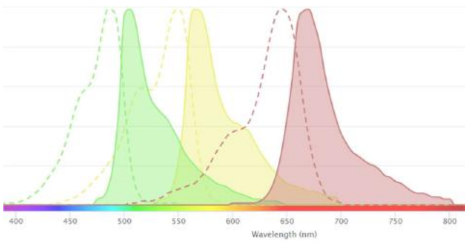
Name	Excitation/Emission (nm)
DAPI	360/460
Propidium Iodide (PI)	488/562-617





a. **Cell Membrane**. Protecting and sequestering the cell interior, the cell membrane is composed primarily of lipids and proteins. In addition to controlling movement across the bilayer, cell membranes are involved in a variety of cellular processes such as cell adhesion, ion conductivity, cell signaling, and extracellular structure attachment.

Name	Excitation/Emission (nm)
DiO/DiL/DiD	DiO 484/501 Dil 549/565 DiD 644/665



b. **Cytoskeleton**. As a complex network of interlinking filaments and microtubules extending throughout the cytoplasm, the cytoskeleton provides structural support to the cell and controls many processes, including endocytosis, cytokinesis, cell migration, invasion, and movement.

Example of staining protocol (MitoTracker Green, selectively targets mitochondria):

1. Wash coverslip twice with PBS.
2. Pippet 100 µL of the MitoTracker Green Working Solution (200 nM) onto the coverslip. Incubate the coverslip for 20 minutes at 37°C, protected from light.
3. Drain off the staining medium and wash the coverslips 2-3 times with PBS, dry.
4. Put a drop of mowiol 5 µL on a glass slide. Make sure that there aren't any bubbles.
5. Take coverslip with tweezers, hold it with the cell surface down and put on mowiol drop while tilted. Label the glass slide.
6. Image using the appropriate emission and excitation filters.

If we want to use more than one dye at a time we have to make it properly. We have to select fluorochrome to avoid emission spectral overlap. To do that, you can use these spectrum viewers: ThermoFisher, BDbiosciences. and AAT.

Part 2. Immunostaining staining

Immunostaining is a technique that uses antibodies conjugated with fluorophore to detect a specific protein in a sample.

So, what is immunostaining? Immunostaining is the term for using antibodies to detect a specific protein in a sample. We will talk about immunofluorescence where antibodies conjugate with fluorophores. First of all, we have to know what antigen is. An antigen is any substance that causes your immune system to produce antibodies against it. So, it’s something “not yours”, strange and maybe dangerous. A specific region of this antigen is called epitope. But is it, antibody (or **immunoglobulin**)? It is a large, Y-shaped protein. There are five **antibody classes** known as IgA, IgD, IgE, IgG, and IgM. The classes differ in their biological properties, functional locations and ability to deal with different antigens. But for our training we need only IgG.

So, we need antibodies to bind specific proteins inside the cell. But why do we need this? We want to find out where a particular protein is expressed, or if it isn't expressed at all, and how many.

There are two types of antibodies available to scientists: polyclonal and monoclonal. **Polyclonal antibodies** contain a heterogeneous mixture of IgGs against the whole antigen, whereas **monoclonal antibodies** are composed of a single IgG against one epitope.

Polyclonal antibodies	**Monoclonal antibodies**
Refer to a mixture of immunoglobulin molecules that are secreted against a particular antigen.	Refer to a homogenous population of antibodies that are produced by a single clone of plasma B cells.
Produced by different clones of plasma B cells.	Produced by the same clone of plasma B cells.
Production does not require hybridoma cell lines.	Production requires hybridoma cell lines.
A heterogeneous antibody population.	A homogenous antibody population.
Interact with different epitopes on the same antigen.	Interact with a particular epitope on the antigen.

There are few steps before staining:

1. Fixation

1. Aspirate the culture medium from the dish or remove each coverslip as required with tweezers, and gently wash them with PBS at room temperature.
2. Incubate the coverslips in freshly prepared 2-4% formaldehyde – neutral PBS at room temperature for 10 minutes. Alternatively, the cells can be fixed for 10 minutes in chilled methanol (pre-equilibrated at -20°C) on ice.

   Tip: Alternative fixation methods (ethanol, methanol or another) may be tested and compared to determine which is best at preserving the structure and epitope of the protein of interest.

3. Wash the coverslips off the fix buffer in PBS for 2 minutes.

2. Permeabilization

1. Incubate the coverslips in 0.5% Triton X-100 in PBS at room temperature for five minutes. Test different detergents (ex. digitonin, Tween-20) in a range of concentrations to find the optimal condition that best preserves cell structure and the target protein.

Tip: A permeabilization step is not required with methanol fixation because methanol acts as a fixative as well as cell permeabilization agent.

2. Wash the coverslips of the permeabilization buffer by incubating in PBS for 5 minutes.

3. Blocking

1. IgG from the secondary antibody may bind non-specifically to the sticky sites on the cells which often leads to non-specific background signals. To avoid this issue, block the coverslips in 1-5% BSA in PBS.

Multicolor labeling experiments are best carried out by sequentially incubating cells with primary and secondary antibodies, however it may be performed by employing one of the following three options:

a) Simultaneous incubation with unlabeled primary antibodies

This method is useful when the primary antibodies are from different hosts. For example, a mouse monoclonal antibody against antigen-X and rabbit polyclonal antibody against antigen Y.

1. After the blocking step, incubate the cells with unlabeled primary antibodies in the blocking buffer in a humidified chamber for 1h at RT or overnight at 4°C.
2. Decant the antibody solution and wash the cells three times in PBS (5 minutes for each wash).
3. Incubate the cells with both secondary antibodies in a blocking buffer for 1h at RT in the dark.

Tip: The secondary antibodies often come with a broad range of working dilutions. It is recommended to choose the dilutions very carefully and to employ additional optimization to see which dilution combination gives the best possible staining.

4. Decant the secondary antibody solution and wash three times with PBS for five minutes each in the dark.

b) Simultaneous incubation with directly labeled primary antibodies

This method is useful when the primary antibodies are from the same host. For example, a mouse monoclonal against antigen-X and a mouse monoclonal against antigen-Y.

1. After the blocking step, incubate the cells with directly labeled primary antibodies in the blocking buffer in a humidified chamber for 1h at RT or overnight at 4°C in the dark.
2. Decant the antibody solution and wash the cells three times in PBS (five minutes for each wash).

Part 3. Live cell staining

The acquisition of information about the “molecular state” of a sample already is a hard task in fixed cells or tissue. This becomes even harder, if the information has to be acquired in real-time, as the cells during an experiment have to be functioning as naturally as possible. Additionally, a high amount of information has to be sampled in a relatively short time, as many events only last seconds or even milliseconds (e.g. changes in cellular ion levels).

Live-cell imaging allows for investigation of dynamical physiological processes in living cells instead of giving a “snapshot” of a cell’s current state. It turns Snapshots into movies. Live-cell imaging provides spatial and temporal information of dynamic molecular events in single cells.

1. It allows the determination of appropriate time points for endpoint studies: many biological processes take a long time (hours to weeks) and often choosing the most significant time points for analysis can be challenging, e.g. when to collect cells after treatment or differentiation, etc. Being able to monitor the cells’ response in real-time enables more accurate identification of relevant endpoints

2. It provides additional and continuous data points of biological processes over time, giving more robust quantitative analysis
3. It enables quantification of transient phenotypic changes in cells that could be missed if only looking at given time points
4. It helps to improve the interpretation of conflicting results from endpoint studies that can be an outcome of reversible processes that are dynamic and controlled in space and time

Successful live-cell imaging experiments can be a major technical challenge. An important caution is to ensure that cells are in good condition and function normally while on the microscope stage with illumination in the presence of synthetic fluorophores or fluorescent proteins. The conditions under which cells are maintained on the microscope stage, although widely variable, often dictate the success or failure of an experiment.

Imaging Media

Various cell culture media are available based on the particular biochemical requirements of cells. Culture media contain various constituents, including amino acids, vitamins, inorganic salts (minerals), trace elements, nucleic acid constituents (bases and nucleosides), sugars, tricarboxylic acid cycle intermediates, lipids, and coenzymes. In tissue culture media, an important step is to control oxygen concentration, pH, buffering capacity, osmolarity, viscosity, and surface tension.

Maintenance of the Cellular Environment in Culture

A constant cellular environment is important to maintain. Cells should be grown in culture medium in a carbon dioxide incubator. For permanent equipment, a box can be constructed around the microscope and heated with warm air.

Choice of Imaging Chamber

Chambers need to keep the specimens viable, while also providing optical properties that are optimal for imaging. Long-term experiments can also be performed for imaging live cells on the microscope stage. The culture cells need to have optimum growth conditions for an extended period of time. In general, imaging chambers include a glass window, usually the thickness of a coverslip (approximately 170 μm), through which the cells are viewed with an objective lens. Short-term experiments can be performed by sandwiching two coverslips.

Phototoxicity

Cells are prone to photodamage, especially when fluorophores are present. When fluorescent molecules are in an excited state, they react with oxygen to produce free radicals that can damage subcellular components and adversely affect the cells. Even when fluorophores are absent, mammalian cells are sensitive to ultraviolet light.

For obtaining the maximum signal/noise ratio and resolution, cells need to be illuminated with very high light intensity. Many methods are used to reduce light-induced damage. An important protective step is to shut off the illuminating light when not. Optimized emission filters should be selected for a maximal signal. Reducing oxygen levels can help limit photobleaching. Additionally, omitting phenol red and serum from the medium can help reduce background fluorescence.

Establishing Cell Morphology and Conditions in Culture

One of the most important aspects of live-cell imaging experiments is to establish criteria for determining the condition of the cells. These criteria will vary depending upon the type of experiment; however, one of the most important factors to determine is whether there is damage to the cells caused by the imaging process that might affect the results. One of the simplest ways for following an imaging experiment is to compare the morphology and condition of cells that were exposed to light during the study with neighboring cells in the same chamber that were not illuminated.

Example of staining protocol (MitoSOX Red selectively targets mitochondria):

1. Wash cells twice with Tyrode's solution.
2. Pipet 100 µL of the MitoSOX Working Solution (1 µM) to the dish. Incubate it for 20 minutes at 37°C, protected from light.
3. Drain off the staining medium and wash the coverslips 2-3 times with Tyrode's.
4. Wash twice with Tyrode's solution.
5. Add 300 µl Tyrode's solution to the dish.
6. After you set up confocal parameters (the appropriate emission and excitation filters, gain, offset etc), add 300 µl 0.5% H_2O_2 as an apoptosis inducer (but this concentration is high, so it induces necrosis).

CELL STAINING QUIZ

1. What is the purpose of cell staining in microscopy?
   1. To enhance the visibility and contrast of cellular structures
   2. To increase magnification
   3. To induce cellular differentiation
   4. To amplify cellular fluorescence during mitosis
   5. All of the above

2. Select the correct statement about the role of primary antibodies in immunostaining
   A. Primary antibodies provide color to the immunostained tissues.
   B. Primary antibodies are used to detect the presence of secondary antibodies.
   C. Primary antibodies bind specifically to the antigen of interest in the tissue
   D. They are always conjugated to a fluorescent molecule

3. What role does the Propidium Iodide stain play in cell imaging?
   A. It stains the cell wall of bacteria
   B. It is a fluorescent stain used for nucleic acids
   C. It visualizes the mitochondria
   D. It stains the cytoplasm

4. What is the main advantage of live cell imaging?
   A. To monitor real-time changes in cellular morphology and behavior
   B. To manipulate cellular gene expression
   C. It permanently stains cells for future observation
   D. To visualize cellular DNA replication in fixed samples
   E. None of the above

5. What is main purpose of using differential interference contrast (DIC) microscopy?
   A. To provide a three-dimensional perspective of the specimen
   B. To visualize the cellular nucleus
   C. To highlight the presence of bacteria
   D. To enhance contrast in unstained, transparent specimens

6. What is the purpose of the secondary antibody in immunofluorescence staining?
   A. It binds directly to the antigen within the cell
   B. It prevents non-specific binding of the primary antibody
   C. It binds to the primary antibody providing a secondary marker such as fluorescent tag
   D. It is used to permeabilize the cell membrane

7. What is the purpose of a counterstain in microscopy?
   A. To stain dead cells
   B. To provide contrast to the primary stain
   C. To reduce background noise in images
   D. To enhance the resolution of nuclear staining

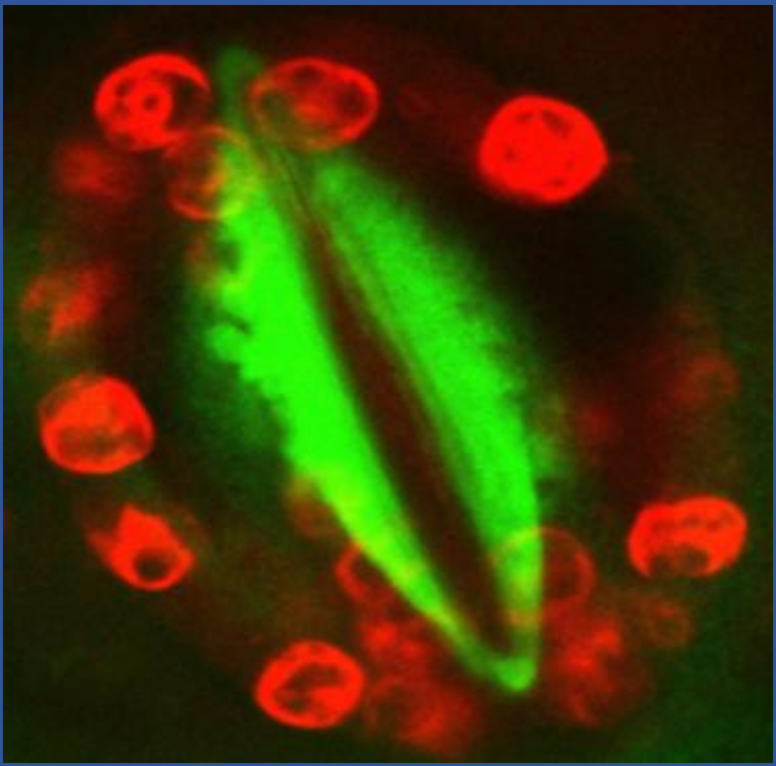
8. The role of blocking solution in immunostaining protocols is
   A. To prevent non-specific binding of antibodies
   B. To increase the permeability of the cell membrane
   C. To activate the fluorescence of the secondary antibody
   D. To dissolve the cell wall for better antibody penetration

9. Select the correct statement in regards to terms 'photobleaching' and 'phototoxicity'

   A. Both can be caused by excessive or prolonged light exposure during imaging.
   B. Photobleaching and phototoxicity are side-effects of light-based imaging techniques.
   C. Phototoxicity refers to cell damage caused by illuminating light.
   D. Photobleaching refers to the reduction in the marker's ability to fluoresce due to light exposure.
   E. All of the above

10. Select the most correct statement related to direct and indirect immunostaining techniques
   A. Direct staining uses fluorescent dyes, while indirect staining uses radioactive tracers.
   B. Direct immunostaining directly labels the target antigen, while indirect immunostaining amplifies the signal through an additional binding step.
   C. Direct immunostaining requires a secondary antibody labeled with a fluorophore, while indirect immunostaining uses a primary antibody labeled with a fluorophore.
   D. Direct immunostaining is faster but less specific than indirect immunostaining.

CONFOCAL MICROSCOPY



INTRODUCTION

Confocal Imaging Concept. The primary functions of a confocal microscope are to produce a point source of light and reject out-of-focus light, which provides the ability to image deep into tissues with high resolution, and optical sectioning for 3D reconstructions of imaged samples. The basic principle of confocal microscopy is that the illumination and detection optics are focused on the same diffraction-limited spot, which is moved over the sample to build the complete image on the detector. While the entire field of view is illuminated during confocal imaging, anything outside the focal plane contributes little to the image, lessening the haze observed in standard light microscopy with thick and highly-scattering samples, and providing optical sectioning.

Manual inverted microscope (model Leica DMi8) with TCS SPE confocal module. It is designed for all common microscope applications and techniques. The microscope is equipped with traditional transmitted light, fluorescence system, three laser lines (488nm, 532nm, 635nm) and tunable emission detector. The system is equipped with LAS X software that allows to process and quantify the acquired images.

Laser Scanning Confocal Microscopy (LSCM) is an advanced optical imaging technique that has revolutionized the way scientists observe and analyze biological samples and materials. Unlike conventional light microscopy, which often suffers from blurred images due to the scattering of light from out-of-focus planes, LSCM provides sharp, high-resolution images by focusing on a single plane within a specimen. This makes it an essential tool for studying the detailed structure of cells, tissues, and various materials.

The fundamental principle behind confocal microscopy is its ability to selectively illuminate and collect light from a specific focal plane within a sample. This is achieved through the use of a laser as the light source and a pinhole aperture that blocks out- of-focus light. As the laser scans across the sample, it excites fluorescent dyes or naturally fluorescent molecules within the specimen. The emitted fluorescence passes through the pinhole and is detected by a photodetector, which constructs an image based on the intensity of the fluorescence at each point.

One of the key advantages of LSCM is its capability to produce optical sections, or "slices," of a specimen. By adjusting the focal plane, the microscope can capture images at different depths, allowing for the reconstruction of three-dimensional structures through a process known as optical sectioning. This is particularly useful in biological research, where understanding the complex architecture of cells and tissues in three dimensions is crucial.

Another significant benefit of LSCM is its ability to reduce background noise and increase contrast in images. This is especially important when working with thick specimens or when imaging multiple layers of cells. The enhanced clarity provided by LSCM allows researchers to distinguish fine details that would be otherwise obscured in traditional microscopy. LSCM is widely used in various fields, including cell biology, neuroscience, developmental biology, and materials science. In cell biology, for instance, it enables the visualization of cellular structures like the nucleus, cytoskeleton, and organelles with high precision. Neuroscientists use LSCM to study the intricate networks of neurons and synapses, while developmental biologists employ it to observe the processes of embryonic development in real-time. In materials science LSCM helps in analyzing surface features, defects, and the distribution of materials in composite structures.

Ներածություն

Կոնֆոկալ միկրոսկոպի հիմնական գործառույթներն են՝ ստեղծել լույսի կետային աղբյուր և բացառել ոչ ֆոկուսային լույսը, ինչը հնարավորություն է տալիս պատկերել հյուսվածքների խորքային շերտերը բարձր լուծաչափով, ինչպես նաև իրականացնել օպտիկական շերտավորում՝ ստացված նմուշների եռաչափ վերակազմության (3D reconstruction) համար: Կոնֆոկալ միկրոսկոպիայի հիմնական սկզբունքն այն է, որ լուսավորման և դետեկտավորման օպտիկական համակարգերը կենտրոնացած են նույն դիֆրակցիոն սահմանափակված կետում (diffraction-limited spot), որը շարժվում է նմուշի մակերեսով՝ դետեկտորի վրա ամբողջական պատկեր ստանալու նպատակով: Կոնֆոկալ պատկերման ընթացքում լուսավորվում է տեսադաշտի ամբողջ հատվածը, սակայն ֆոկուսային հարթությունից դուրս գտնվող տարրերը նվազ չափով են մասնակցում պատկերի ձևավորմանը՝ նվազեցնելով մթնշաղային աղավաղումները, որոնք բնորոշ են սովորական լուսային միկրոսկոպիայում հաստ և բարձր ցրման հատկությամբ նմուշների դեպքում, և ապահովելով օպտիկական շերտավորում:

Ձեռքով կառավարվող (մանուալ) շրջված միկրոսկոպ (**մոդել Leica DMi8**)՝ համալրված **TCS SPE կոնֆոկալ մոդուլով**, նախատեսված է միկրոսկոպիայի հիմնական կիրառությունների և տեխնիկաների լայն շրջանակի համար: Միկրոսկոպը համալրված է ավանդական անցնող լույսի համակարգով, ֆլուորեսցենտային պատկերավորման մոդուլով, երեք լազերային գծերով՝ **488 նմ, 532 նմ և 635 նմ,** ինչպես նաև **կարգավորվող արտանետման դետեկտորով**: Համակարգը համալրված է **LAS X ծրագրային ապահովմամբ**, որը հնարավորություն է տալիս ստացված պատկերների մշակում և քանակական վերլուծություն իրականացնել:

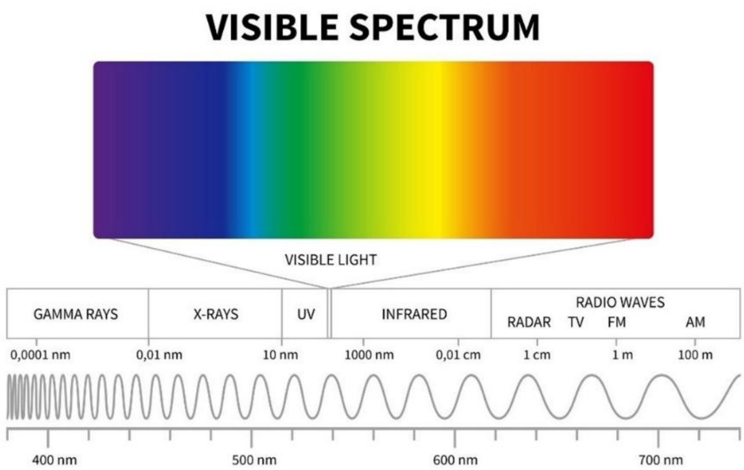
Լազերային սկանավորման կոնֆոկալ մանրադիտակումը (LSCM) առաջադեմ օպտիկական պատկերավորման տեխնիկա է, որը արմատապես փոխակերպել է գիտնականների կողմից կենսաբանական նմուշների և նյութերի դիտման ու վերլուծության մեթոդները: Ի տարբերություն դասական լուսային մանրադիտակման, որը սահմանափակվում է ոչ ֆոկուսային շերտերից ցրված լույսի հետևանքով առաջացած աղոտ պատկերներով, **LSCM-ը** ապահովում է հստակ և բարձր լուծաչափով պատկերներ՝ կենտրոնանալով նմուշի մեկ ֆոկուսային շերտի վրա: Այս հատկությունը դարձնում է այն անփոխարինելի գործիք բջիջների, հյուսվածքների և տարբեր նյութերի մանրակրկիտ կառուցվածքային ուսումնասիրության համար:

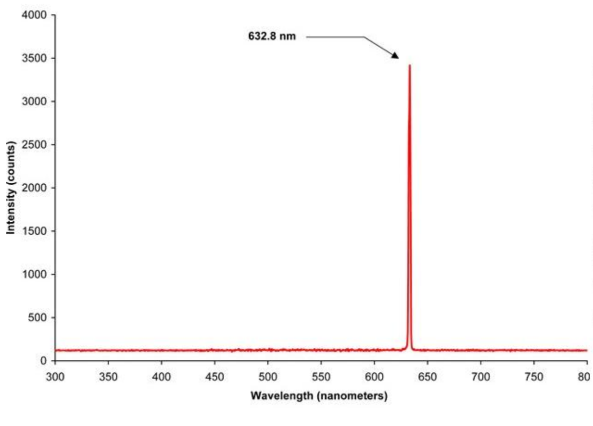
Կոնֆոկալ մանրադիտակման հիմնական սկզբունքը հիմնված է նմուշի որոշակի ֆոկուսային հարթությունից լույսը ընտրովի կերպով լուսավորելու և հավաքելու կարողության վրա: Սա իրականացվում է լազերի՝ որպես լույսի աղբյուրի օգտագործմամբ և փոքր անցքով ապերտուրայի միջոցով, որը արգելափակում է ոչ ֆոկուսային լույսը: Լազերային ճառագայթը սկանավորելով նմուշը՝ գրգռում է նմուշում առկա ֆլուորեսցենտ ներկանյութերը կամ բնական ֆլուորեսցենտ մոլեկուլները: Գրգռման արդյունքում առաջացած ֆլուորեսցենտ ճառագայթումը անցնում է ապերտուրայով և գրանցվում է ֆոտոդետեկտորի կողմից, որը կառուցում է պատկերը՝ հիմնվելով յուրաքանչյուր կետում ֆլուորեսցենտի ինտենսիվության վրա:

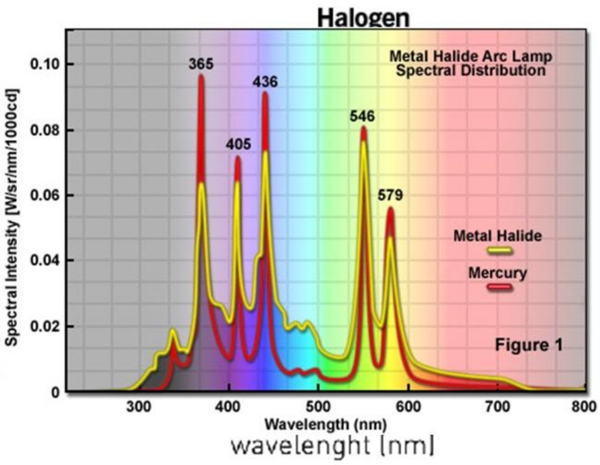
LSCM-ի հիմնական առավելություններից մեկը նմուշի օպտիկական շերտերի կամ «կտրվածքների» (optical sections) ստացման հնարավորությունն է: Ֆոկուսային հարթության փոփոխման միջոցով միկրոսկոպը կարող է պատկերներ ստանալ տարբեր խորությունների վրա՝ հնարավորություն տալով վերականգնել եռաչափ կառուցվածքներ օպտիկական շերտավորման միջոցով: Այս մեթոդը հատկապես արժեքավոր է կենսաբանական հետազոտություններում, որտեղ բջիջների և հյուսվածքների բարդ եռաչափ կառուցվածքի ըմբռնումը վճռորոշ նշանակություն ունի:

LSCM-ի ևս մեկ կարևոր առավելությունը պատկերի ֆոնի նվազեցումն է և կոնտրաստի բարձրացումը, ինչը հատկապես կարևոր է հաստ նմուշների կամ բջջային շերտերի բազմաշերտ պատկերավորման դեպքում: Բարձր հստակությունը հնարավորություն է տալիս տարբերակել մանրագույն կառուցվածքային տարրեր, որոնք սովորական միկրոսկոպիայում կմնային անտեսանելի: LSCM-ը լայնորեն կիրառվում է տարբեր ոլորտներում՝ ներառյալ **բջջային կենսաբանությունը, նյարդաբանությունը, զարգացական կենսաբանությունը և նյութագիտությունը:** Բջջային կենսաբանության մեջ այն հնարավորություն է տալիս բարձր ճշգրտությամբ դիտարկել բջջային կառուցվածքներ, ինչպես օրինակ՝ միջուկը, ցիտոսկելետը և օրգանոիդները: Նյարդաբանության մեջ այն կիրառվում է նեյրոնների և սինապսների բարդ ցանցերի ուսումնասիրման համար, մինչդեռ զարգացական կենսաբանության մեջ օգտագործվում է սաղմնային զարգացման պրոցեսների իրական ժամանակում դիտարկման նպատակով: Նյութագիտության մեջ LSCM-ը օգնում է վերլուծել մակերեսային առանձնահատկությունները, թերությունները և նյութերի բաշխվածությունը կոմպոզիտային կառուցվածքներում:

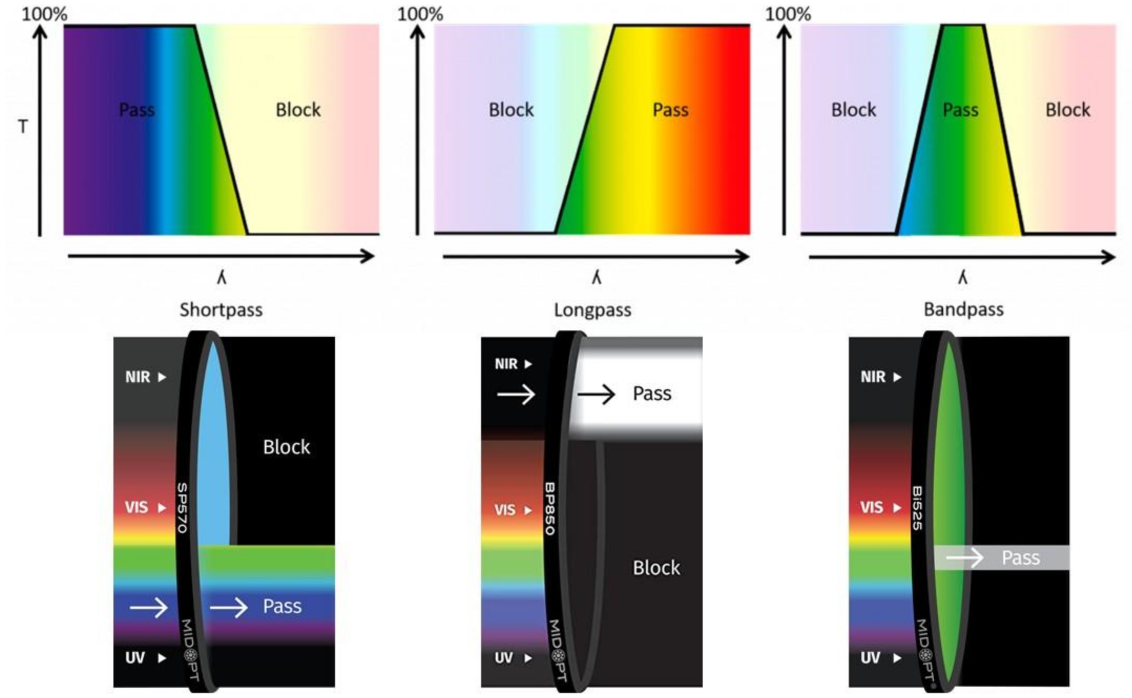
PROTOCOLS AND VISUALS

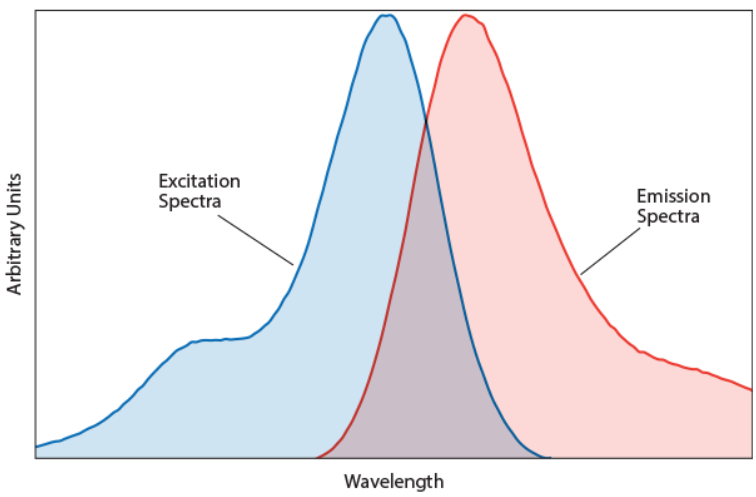


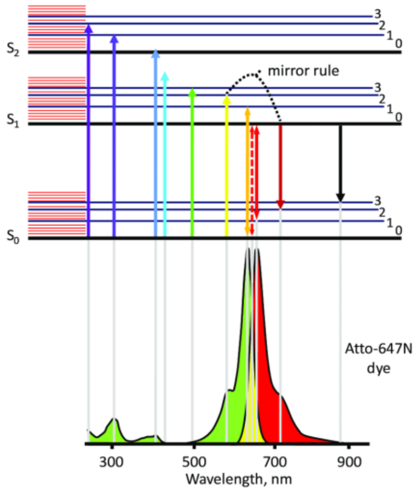




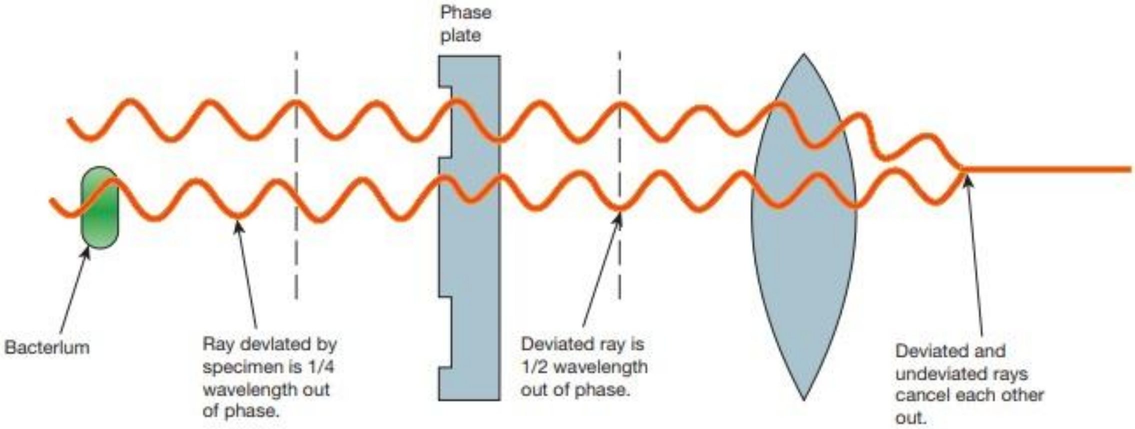
Filters

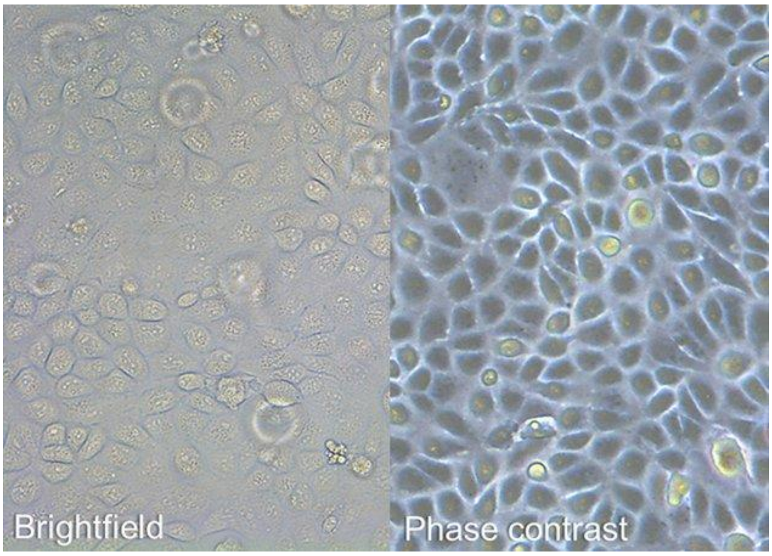

100%
T
Pass
Block
Shortpass
Block
Pass
Longpass
Block
Pass
Block
Bandpass
NIR
VIS
UV
SP570
BP850
Bi525
Pass
Block


Arbitrary Units
Excitation
Spectra
Emission
Spectra
Wavelength


S_2
S_1
S_0
mirror rule
3
2
1
0
Atto-647N
dye
300
500
700
900
Wavelength, nm

Phase contrast microscopy





Differential Interference Contrast (DIC) microscopy

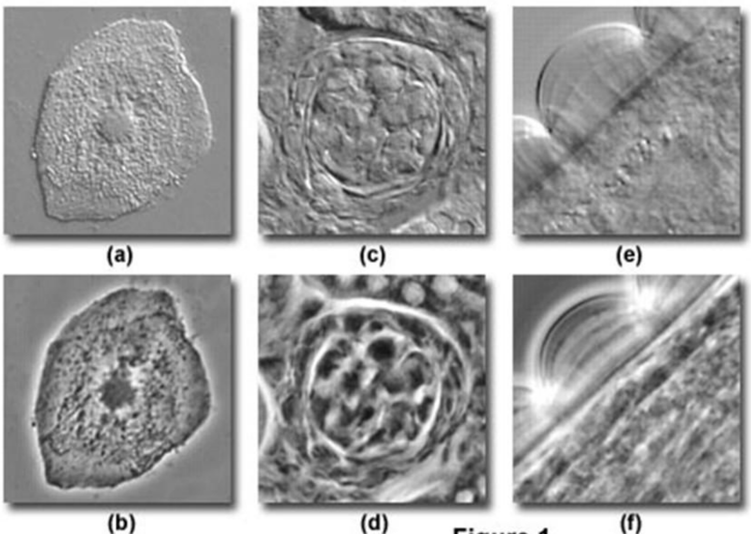
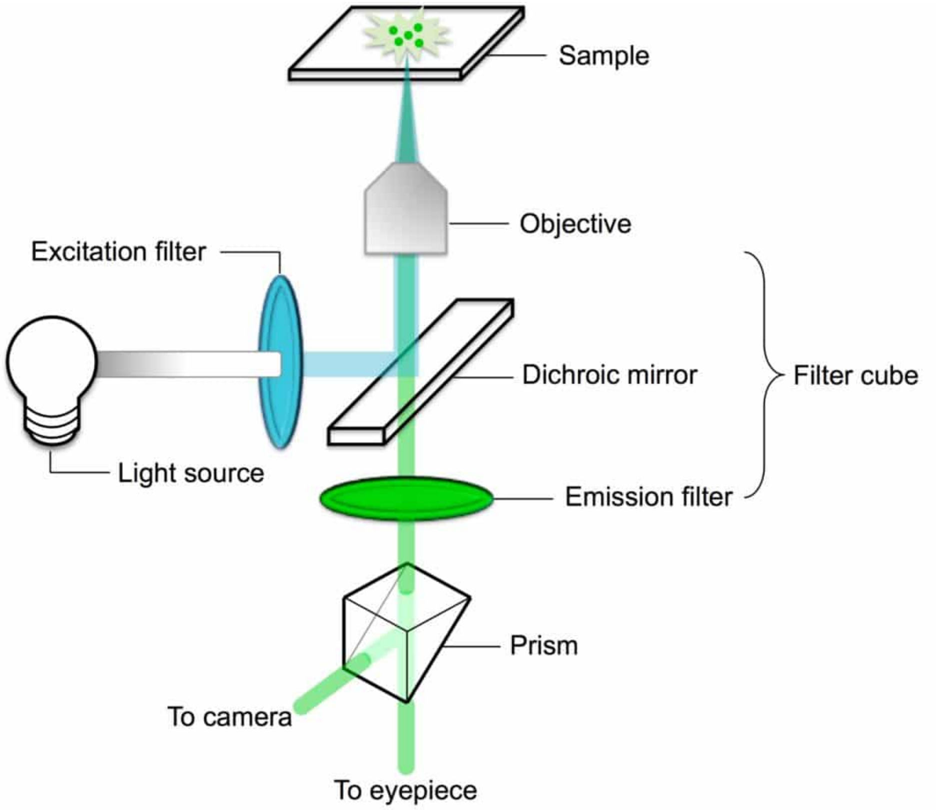
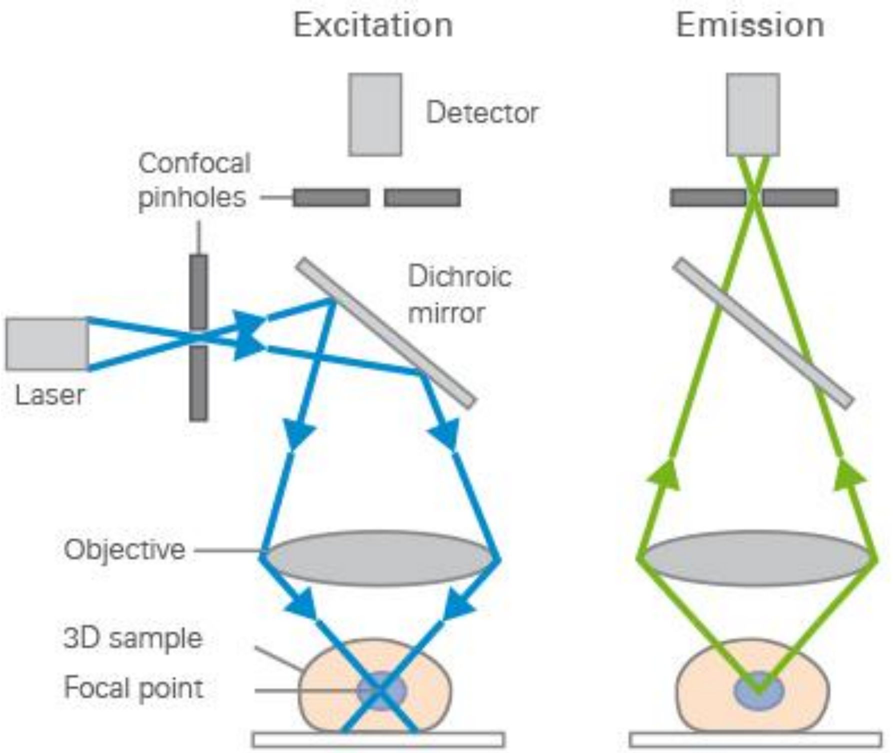


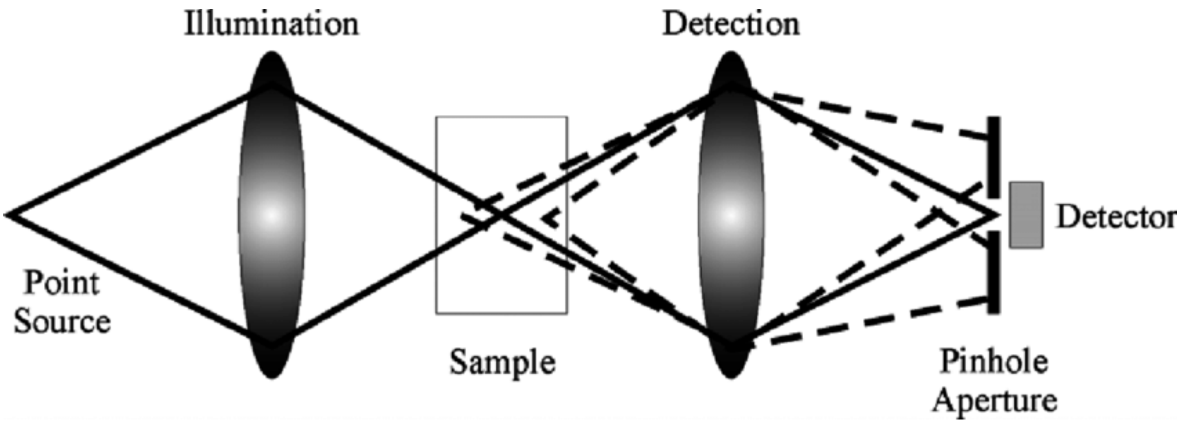
Figure 1

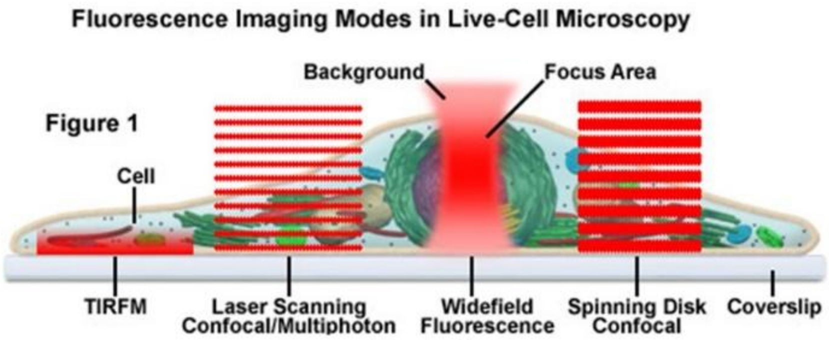
Fluorescence microscopy

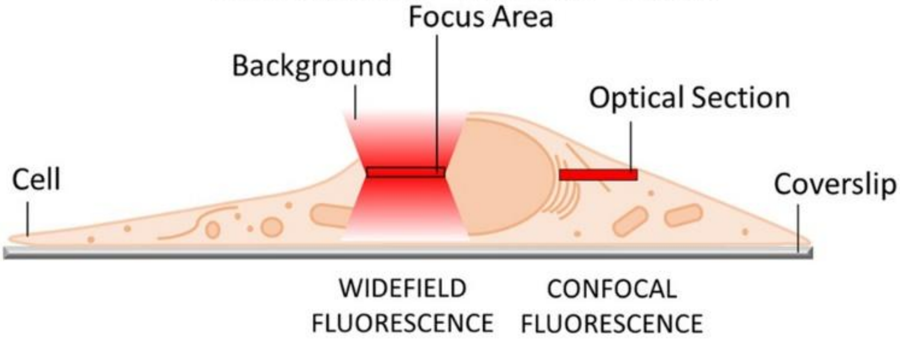


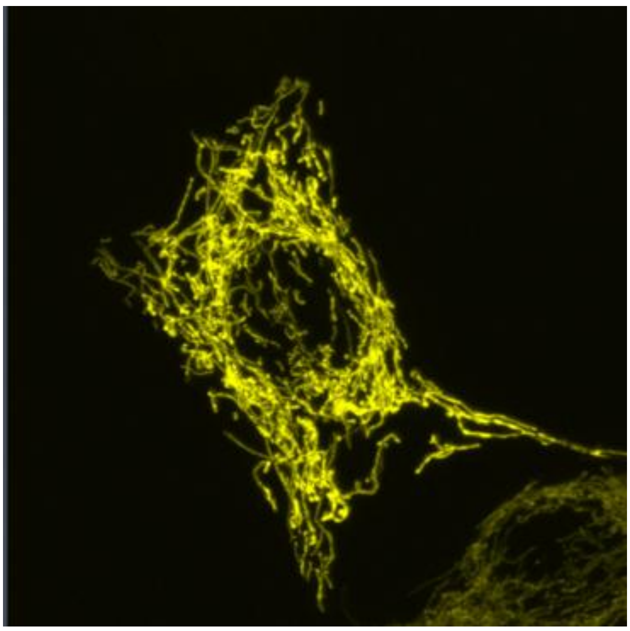
Confocal microscopy

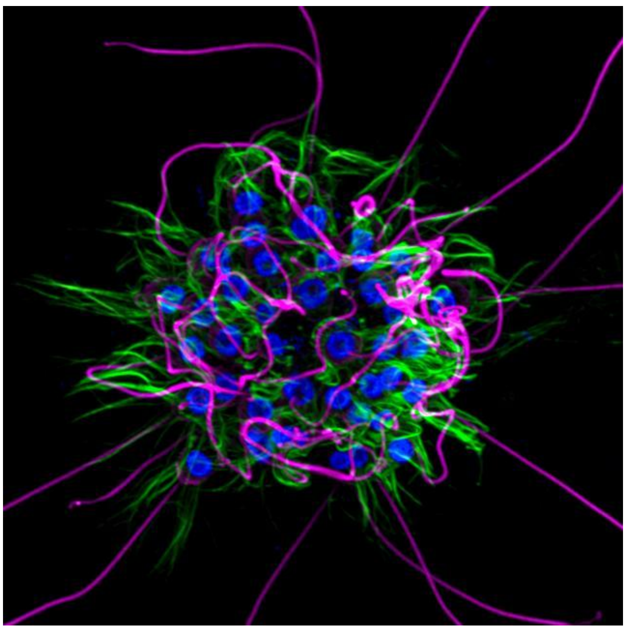



Illumination
Detection
Point
Source
Sample
Pinhole
Aperture
Detector

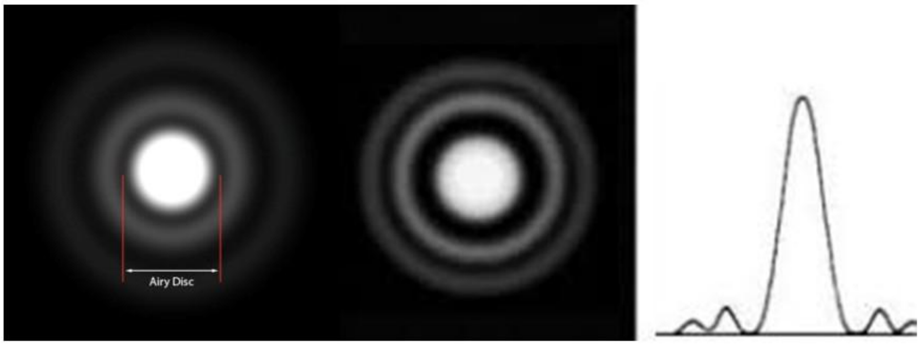

Fluorescence Imaging Modes in Live-Cell Microscopy
Background
Focus Area
Figure 1
Cell
TIRFM
Laser Scanning
Confocal/Multiphoton
Widefield
Fluorescence
Spinning Disk
Confocal
Coverslip

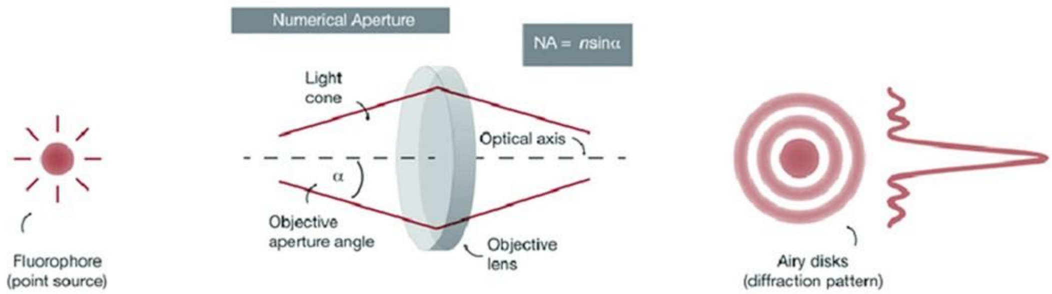

Focus Area
Background
Optical Section
Cell
Coverslip
WIDEFIELD
FLUORESCENCE
CONFOCAL
FLUORESCENCE

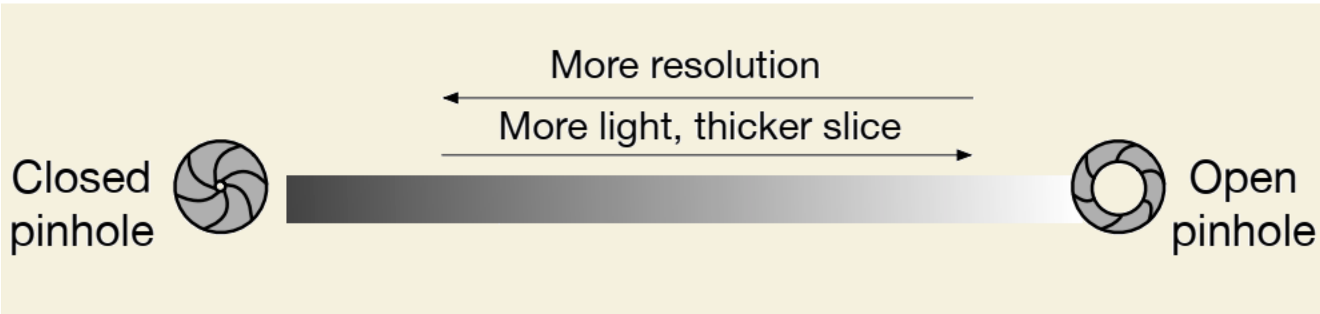


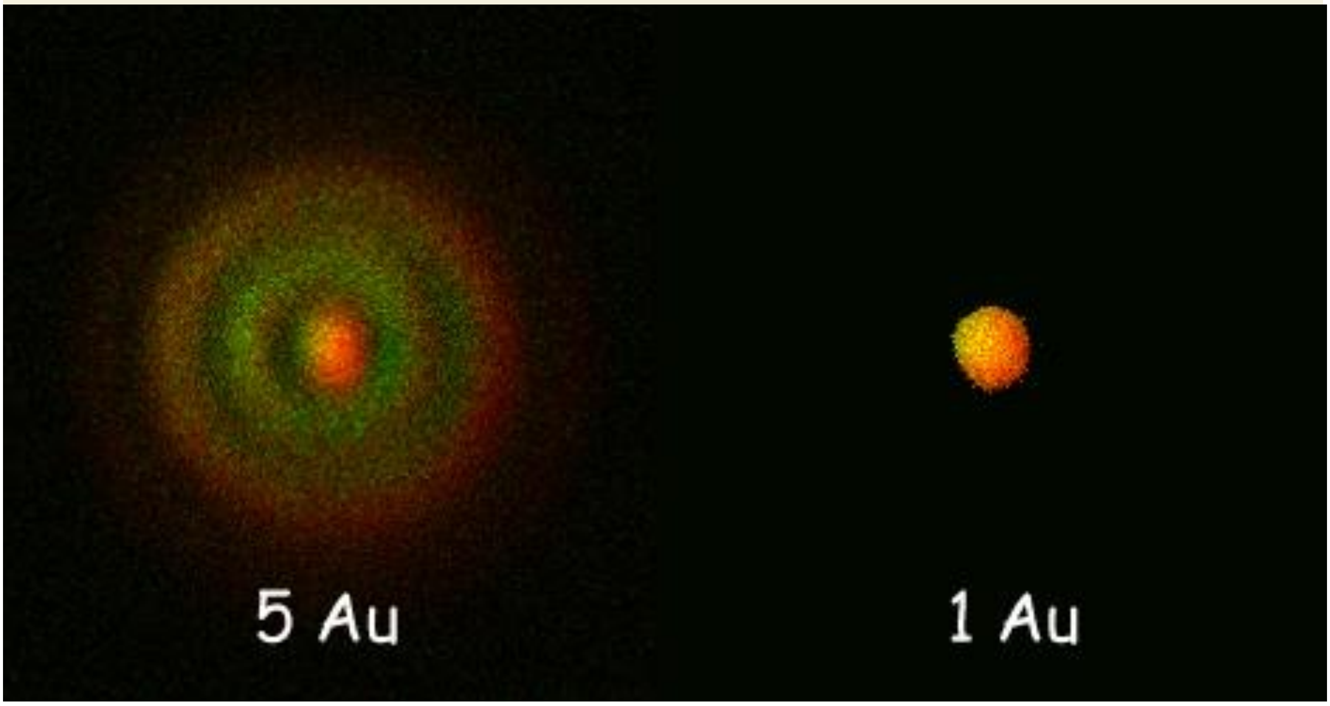


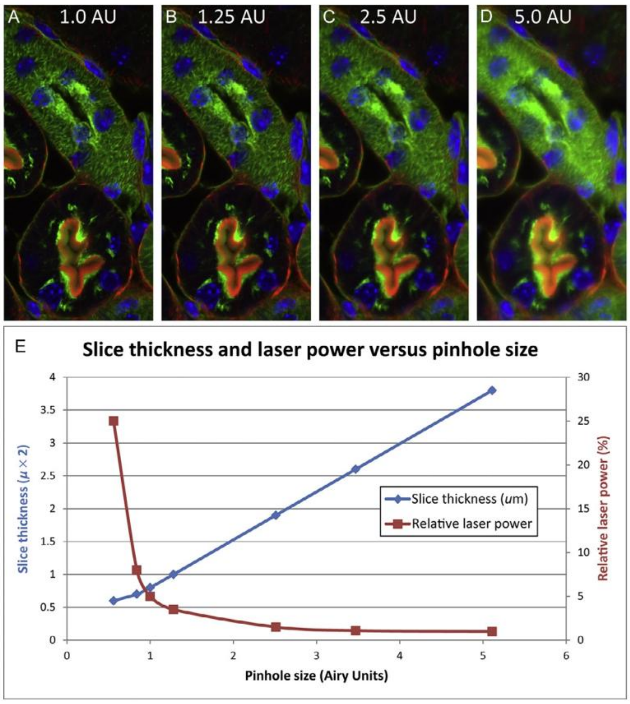
Point Spread Function (PSF), Airy disc

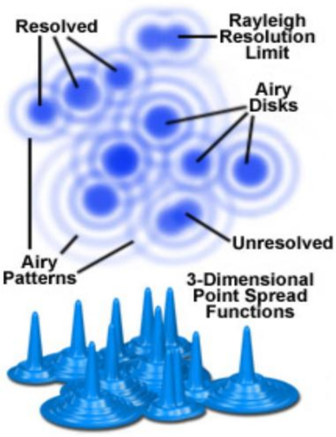


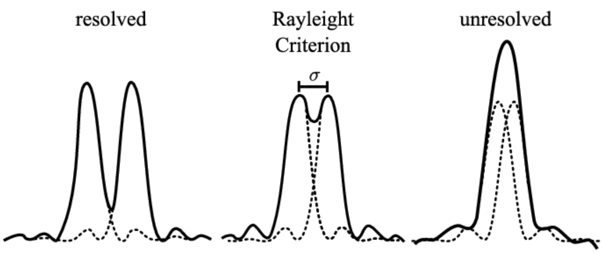




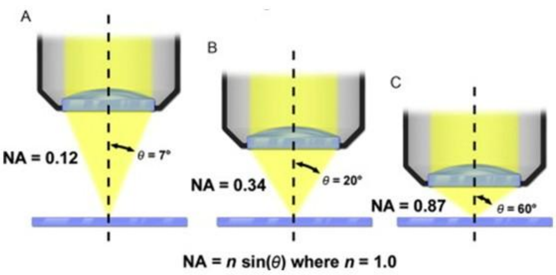


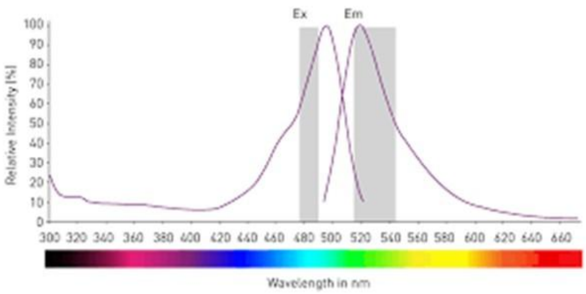






Resolution and Numerical Aperture





For a confocal system, the pinhole radius is set somewhat smaller than or equal to r_{airy} and lateral resolution: **Res XY ≈ 0.4λ_{em}/NA**

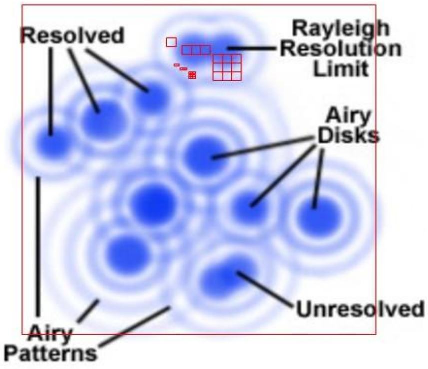
Axial resolution: **Res Z ≈ 1.4$\lambda_{em}\eta$/NA^2**
Wavelength of light (λ), the Numerical Aperture of the lens (NA),
Refractive Index of the medium between the lens and the specimen (η).

Optimizing resolution on a laser scanning confocal

Key concept #3

Optimal sampling in XY and Z

XY: pixel size



Optimal pixel size ~ $\frac{R_{xy}}{2.5}$

"Nyquist criterion"

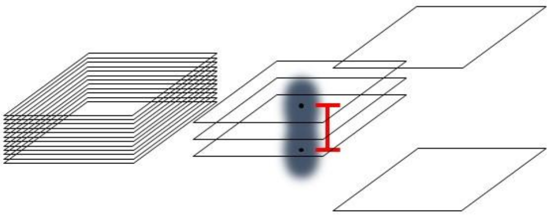
Nyquist Sampling dictates that to optimally represent an analogue signal in digital space, the analogue signal needs to be sampled at least 2.5 times. In microscopy terms this means that the pixel size of an image needs to be at least 2.5 times smaller than the object that is being resolved.

Optimizing resolution on a laser scanning confocal

Key concept #3

Optimal sampling in XY and Z

Z: spacing between slices



Optimal interval spacing ~ $\frac{R_z}{2.5}$ "Nyquist criterion"

CONFOCAL MICROSCOPY QUIZ

1. Laser scanning confocal microscopy is a type of...

A. Light microscopy
B. Electron microscopy
C. Atomic force microscopy
D. X-ray microscopy

2. What is the fundamental principle of laser scanning confocal microscopy?

A. Synchronizing light wavelength with a sample to improve image resolution.
B. Lighting a specific plane of a sample and collecting the same plane's light to reduce out-of-focus signals to achieve better image detail.
C. Illumination of the entire specimen at once
D. Simultaneous excitation of multiple fluorophores

3. How does confocal microscopy enhance the resolution and contrast of a sample?

A. By employing multiple light sources
B. By illuminating the entire specimen at once
C. By focusing a point of light on the specimen and collecting only in-focus light
D. By using multiple detectors

4. What part of the confocal microscope helps to reject out-of-focus light?

A. Dichroic mirror
B. Pinhole
C. Objective lens
D. Light source

5. Which of the following is a primary advantage of confocal microscopy over conventional fluorescence microscopy?

A. Capable of imaging specimens in liquid environments.
B. Faster image production speed.
C. Capability to produce high-resolution images of individual layers within thick samples.
D. Cheaper cost.

6. Confocal microscopy is often used in combination with which technique to produce three-dimensional images?

A. Dark field illumination
B. Phase contrast
C. Electron microscopy
D. Z-stacking

7. When intending to resolve small details using confocal microscopy, which type of fluorophore would be the most appropriate?

A. A fluorophore with a long emission wavelength with a high quantum yield

B. A fluorophore with a short emission wavelength with a high quantum yield
C. A fluorophore with a long emission wavelength with a low quantum yield
D. A fluorophore with a short emission wavelength with a low quantum yield

8. What primarily determines the lateral and axial resolutions in a confocal microscope?

A. The type of specimen being studied
B. The magnification of the ocular lenses
C. The numerical aperture of the objective lens and the wavelength of the light
D. The speed at which the image is converted into digital format

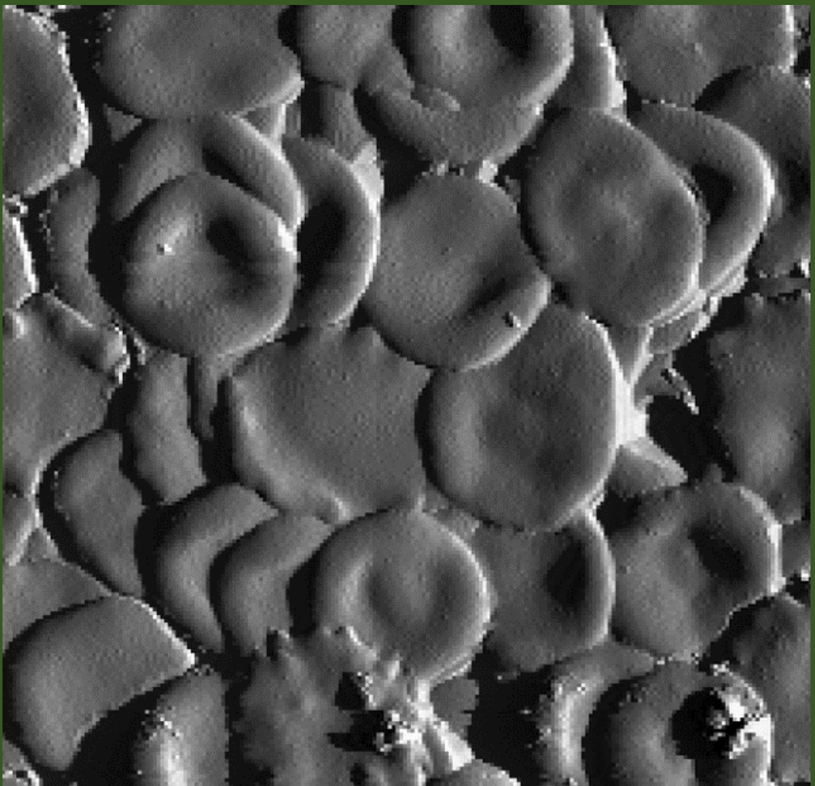
9. What is the main function of the detector in a confocal microscope?

A. To produce the illuminating photons
B. To measure the emitted photons
C. To focus the light
D. To reject out-of-focus light

10. What type of samples are suitable for confocal microscopy?

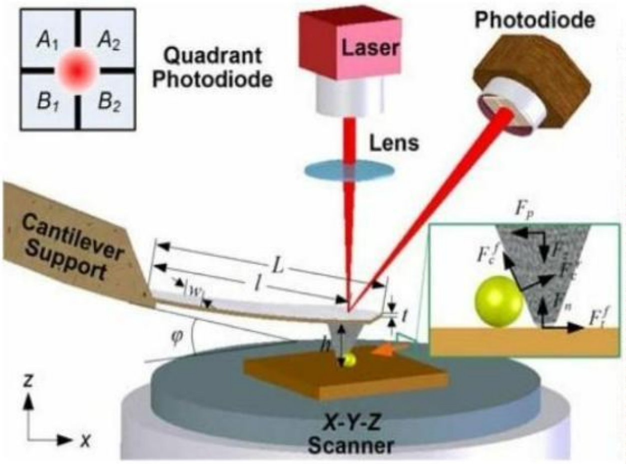
A. Thick samples, such as tissue sections or whole-mount preparations
B. Living cell and tissue imaging
C. Samples that require high-resolution and high-contrast images
D. Samples requiring 3D reconstruction
E. All of the above

ATOMIC FORCE MICROSCOPY

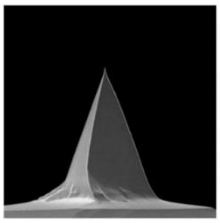


INTRODUCTION

AFM Concept. Atomic Force Microscopy is a very high-resolution type of scanning probe microscopy with demonstrated resolution on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit. The information is gathered by "feeling" or "touching" the surface with a mechanical probe. Piezoelectric elements that facilitate tiny but accurate and precise movements on (electronic) command enable precise scanning. The AFM`s three major abilities: force measurement, topographic imaging, and manipulation, make it a powerful method of investigation and visualization of various life science targets. Additional information about the basics of AFM can be found on [wikipedia page] or by watching this [youtube video.]



AFM Workshop system available at OIPH. The LS-AFM is a tip-scanning AFM designed specifically for life science applications when paired with an inverted fluorescent microscope. The product includes everything required for AFM scanning: AFM Stage, Inverted Microscope Adaptation Plate, Ebox, Manuals, Cables, and AFM-Control Software. The LS-AFM is designed for the most widely used types of measurements made with an AFM, including measuring Force/Distance curves and imaging cells in a dry and liquid environment. [PDF manual.]



In an AFM (atomic force microscope), a probe is scanned over a surface and the motion of the probe is monitored to create a three-dimensional image of the surface. These unique instruments are capable of measuring high-resolution images in both ambient air and liquids, with surface features of only a few nanometers in size. The three-dimensional motion of the sample (or probe) is generated by piezoelectric ceramics. These sensitive ceramics allow motions as small as a fraction of a nanometer. Typically, the sample (or probe) is moved in a raster pattern as the probe glides across the surface. A light lever sensor is used for controlling the force of the probe on the surface while the sample is scanned. The light lever reflects a laser beam off the surface of a cantilever into a photo-detector. As the probe interacts with a surface, the cantilever deflects, and this motion is sensed by the photo-detector. With this light lever, forces as small as a pico-newton are possible. With such small forces, very small probes may be used. With micro-machining methods probes can have diameters of only a few nanometers.The light lever can be made more sensitive by vibrating the cantilever with a small piezoelectric ceramic and modulating the light. When the vibrating probe interacts with the surface, the amplitude of vibration may be monitored and used to control the probe's force on the surface.

Modern atomic force microscopes include not only a probe and piezoelectric scanner, but additional hardware for bringing the probe rapidly into the proximity of a surface. A video optical microscope is very helpful for operating an AFM. The video microscope helps with aligning the light lever and probe approach, and for finding features for scanning. For an in-depth description of AFM instrumentation, we recommend the book Atomic Force Microscopy by Peter Eaton and Paul West. This book provides a complete theoretical, as well as practical explanation for the design and application of AFMs.

Stage. Samples are held and scanned on the AFM stage. On the upright inside the stage is a linear translator which moves both the light lever force sensor and the piezoelectric scanner in a vertical direction. The stage also includes a base plate fitted with precision XY translators. Optimal images are measured with the AFM stage if it is in a vibration- and acoustic-free environment. If necessary, a vibration and acoustic isolation system should be used. On the back cover of the stage is a modes connector. Signals required for implementing additional modes such as conductive AFM, STM, and EFM are provided.

Ներածություն

AFM հասկացությունը: Ատոմային ուժային մանրադիտակը սկանավորող զոնդային մանրադիտակի շատ բարձր լուծաչափով տեսակ է՝ նանոմետրի կոտորակների կարգի լուծաչափով, որը ավելի քան 1000 անգամ ավելի ուժեղ է, քան օպտիկական դիֆրակցիայի սահմանը: Տեղեկատվությունը հավաքվում է մակերեսը մեխանիկական զոնդով «զգալով» կամ դիպչելով, հպվելով: Պիեզոէլեկտրական տարրերը, որոնք հեշտացնում են (էլեկտրոնային) հրամանով փոքր, բայց ճշգրիտ շարժումները, հնարավորություն են տալիս գերճշգրիտ սկանավորել: AFM-ի երեք հիմնական հնարավորությունները՝ ուժի չափումը, տեղագրական պատկերումը և մանիպուլյացիան, այն դարձնում են տարբեր կենսաբանական գիտական թիրախների հետազոտման և վիզուալիզացիայի հզոր մեթոդ: AFM-ի հիմունքների մասին լրացուցիչ տեղեկություններ կարող եք գտնել Վիքիպեդիայի էջում կամ դիտելով այս տեսանյութը :

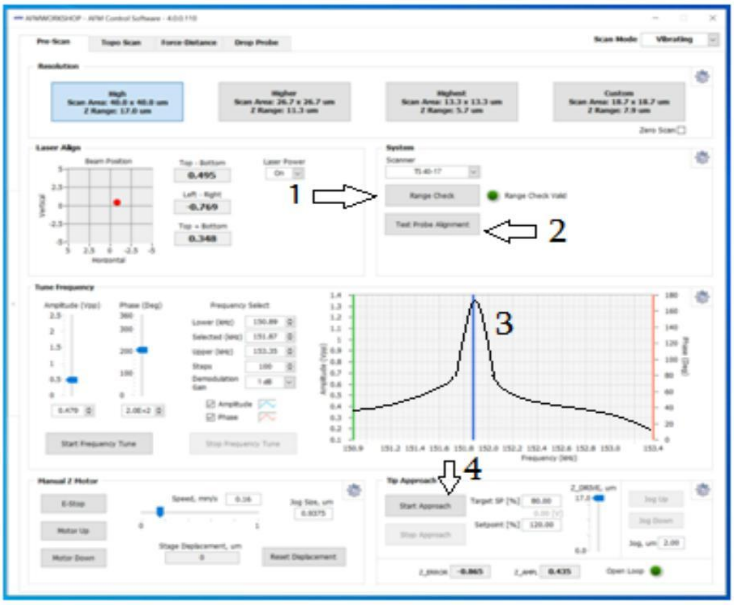
AFM համակարգը հասանելի է Օրբելու անվան Ֆիզիոլոգիայի ինստիտուտում: LS-AFM-ը ծայրային զոնդով սկանավորող AFM է, որը նախագծված է հատուկ կենսաբանական գիտությունների կիրառությունների համար, երբ զուգակցվում է շրջված ֆլուորեսցենտային մանրադիտակի հետ: Արտադրանքը ներառում է AFM սկանավորման համար անհրաժեշտ ամեն ինչ՝ AFM փուլ, շրջված մանրադիտակի ադապտացիոն ափսե, էլեկտրոնային տուփ, ձեռնարկներ, մալուխներ և AFM-կառավարման ծրագիր: LS-AFM-ը նախատեսված է AFM-ով կատարվող ամենատարածված չափումների տեսակների համար, ներառյալ ուժի/հեռավորության կորերի չափումը և չոր և հեղուկ միջավայրում բջիջների պատկերումը: PDF ձեռնարկ:

AFM-ում (ատոմային ուժային մանրադիտակ) զոնդը սահում է մակերեսի վրայով, և զոնդի շարժումը գրանցվում է՝ մակերեսի եռաչափ պատկեր ստեղծելու համար: Այս եզակի սարքերը կարող են չափել բարձր կետայնությամբ պատկերներ՝ ինչպես չոր նմուշի վրա, այնպես էլ հեղուկներում, որոնց մակերեսային առանձնահատկությունները ընդամենը մի քանի նանոմետր են: Նմուշի (կամ զոնդի) եռաչափ շարժումը ստեղծվում է պիեզոէլեկտրական կերամիկայի միջոցով: Այս զգայուն կերամիկան թույլ է տալիս շարժումներ կատարել մինչև նանոմետրի հարյուրերորդական մասը: Սովորաբար, նմուշը (կամ զոնդը) շարժվում է լազերային նախշով, երբ զոնդը սահում է մակերեսի վրայով: Լույսի լծակի սենսորն օգտագործվում է զոնդի ուժը մակերեսի վրա կառավարելու համար, մինչ նմուշը սկանավորվում է: Լույսի լծակը լազերային ճառագայթը արտացոլում է զոնդի մակերեսից դեպի ֆոտոդետեկտոր: Երբ զոնդը փոխազդում է մակերեսի հետ, զոնդը շեղվում է, և այս շարժումը զգացվում է ֆոտոդետեկտորի կողմից: Այս լույսի լծակի միջոցով հնարավոր են դառնում պիկո-նյուտոնի կարգի փոքր ուժերի վիզուալիզացիան: Նման փոքր ուժերի դեպքում կարող են օգտագործվել շատ փոքր զոնդեր: Միկրոմշակման մեթոդներով զոնդերը կարող են ունենալ ընդամենը մի քանի նանոմետր տրամագիծ:

Լույսի լծակը կարելի է ավելի զգայուն դարձնել՝ զոնդին փոքր պիեզոէլեկտրական կերամիկակայով տատանողական շարժում հաղորդելով և լույսը մոդուլացնելով: Երբ տատանվող զոնդը փոխազդում է մակերեսի հետ, տատանման ամպլիտուդը կարող է վերահսկվել և օգտագործվել զոնդի հպման ուժը մակերեսի վրա կառավարելու համար: Ժամանակակից ատոմային ուժային մանրադիտակները ներառում են ոչ միայն զոնդ և պիեզոէլեկտրական սկաներ, այլև լրացուցիչ սարքավորումներ՝ զոնդը մակերեսին արագ մոտեցնելու համար: Տեսաօպտիկական մանրադիտակը շատ օգտակար է AFM-ը գործարկելու համար: Տեսամանրադիտակը օգնում է լույսի լծակի և զոնդի մոտեցումը համապատասխանեցնելուն և սկանավորման համար թիրախների հայտնաբերմանը: AFM գործիքավորման խորը նկարագրության համար մենք խորհուրդ ենք տալիս Պիտեր Իթոնի և Փոլ Ուեսթի «Ատոմային ուժային մանրադիտակ» գիրքը: Այս գիրքը տրամադրում է AFM-ների նախագծման և կիրառման ամբողջական տեսական, ինչպես նաև գործնական բացատրություն:

Հարթակ: Նմուշները պահվում և սկանավորվում են AFM հարթակի վրա: Հարթակի վրա ուղղահայաց դիրքով տեղադրված է գծային փոխադրիչ, որը շարժում է և՛ լույսի աղբյուրը ֆոդոընկալիչի հետ միասին, և՛ պիեզոէլեկտրական սկաները ուղղահայաց ուղղությամբ: Հարթակը նաև ներառում է ճշգրիտ XY փոխադրիչներով հագեցած հիմք: Օպտիմալ պատկերները չափվում են AFM հարթակի միջոցով, եթե այն գտնվում է տատանողական և ակուստիկ աղմուկից զերծ միջավայրում: Անհրաժեշտության դեպքում պետք է օգտագործվի աղմուկի մեկուսացման համակարգ: Հարթակի հետևի կափարիչին ռեժիմների միակցիչ է: Լրացուցիչ ռեժիմների, ինչպիսիք են հաղորդիչ AFM-ը, STM-ը և EFM-ը, իրականացման համար անհրաժեշտ ազդանշանները կարող են արտահանվել:

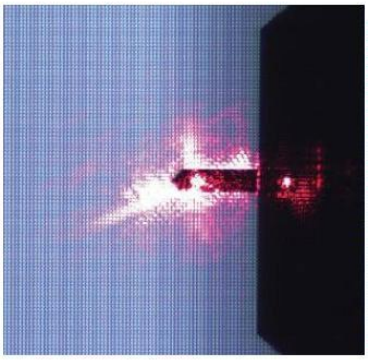
PROTOCOLS AND VISUALS



Software The TT-2 AFM includes three separate software modules in the AFM Installation files: AFM Workshop Acquisition Software, Video Microscope Software, and Gwyddion ImageAnalysis Software.

Gwyddion Image Analysis Software.
Gwyddion is open-source software and the latest version of this image-analysis softwareis available on the Internet at: http://gwyddion.net/. The functions of the Gwyddion imageanalysis software are:
1. Processes such as leveling, deglitching, and smoothing which alter the images.
2. Display functions which change how the data is viewed, including 2-D, 3-D, light shading, and color mapping.
3. Analysis options that are used for obtaining measurements from images, such as line profiling and histogram analysis.

AFM-View Software. Once launched, the AFM-View software has four screens that can be viewed by pressing the tabs at the op right-hand side of the screen. The first tab is for the Pre-Scan window and the second tab is for the
Scan window. These two windows are all that are needed for measuring AFM images. The third tab labeled System is used for several other functions, such as measuring the Z noise floor and XYZ scanner calibration. There is a fourth tab that, when activated, permits force-distance curve measurements.
Working. Before starting the work with the scanner and the software, you need to Laser Align.



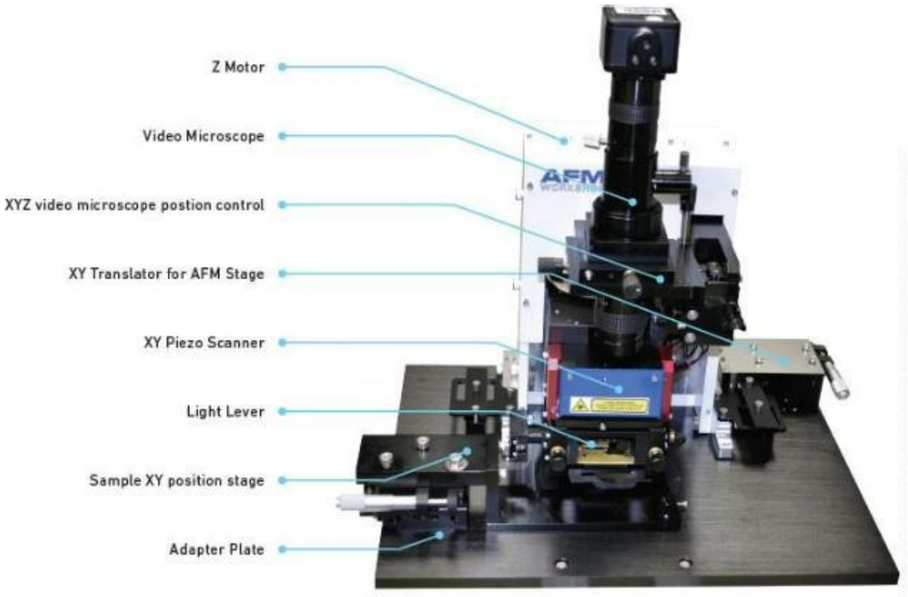
1.1 / LS-AFM Users Guide

1. Switch ON the EBOX

2. Switch ON the PC

3. Enter to the AFM Control software 

4. Press **Range Check** button (1)
5. Once The **Range Check** Button is Valid, **Press Test Probe Alignment** button (2) (for non vibrating mode jump to the step 8)
6. Press **Start Frequency Tune** button and find/fix (move **blue line**) the highest amplitude from the histogram table
7. Change the frequency range to find the pic (move **green** and **red lines**) (3) If the blue line is on the top of the pick (on highest rate of amplitude)
8. Press **Start Approaching** button (4)
9. When the **Open Loop** button is on (**on feedback**) , use **Jog Up** and **Jog Down** buttons to stabilize the z-drive (cursor must be in the middle)
10. Go to **Topo Scan** window
11. Chose the picture quality, number of lines and the scanning speed
12. Press **Start Topo-Scan** button.
13. During the scanning, use GPYD controller to get the best quality.
14. When scanning end, saving window will open automatically. Change the name (if needed), chose the place (folder) and press **Save.**
15. In **Topo-Scan** window press **Tip Retract** button.
16. When the **Tip** is **Retracted,** You can go to **Pre-Scan** window, or close the **Software.**
17. **Switch off the E-Box.**
18. **Switch off the light controller.**



For more detailed review, you can see the following links:
https://www.afmworkshop.com/afm-products/atomic-force-microscopes/ls-afm?srsltid=AfmBOopIxDEN4tWKZoFOhXhNv4cJQHcb1RW5iUxOYJJ3V_1pXNzKiok5
https://afmhelp.com/docs/manuals/10-1121-16%20LS-AFM%20Manual%20v1.1.pdf
For Measuring and Understanding Force Distance Curves
https://www.afmworkshop.com/images/datasheets/Measuring-and-understanding-force-distance-curves-v2.pdf

WHAT IS AFM?

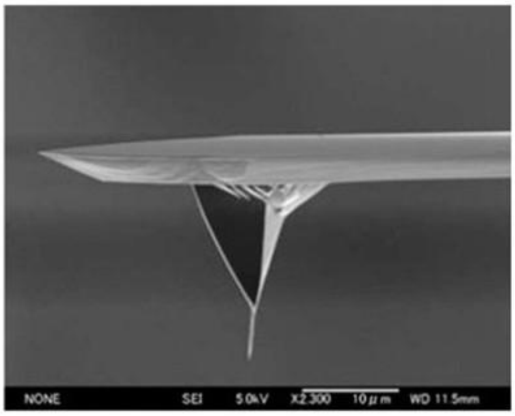
- Atomic force microscopy(AFM) is one of the foremost tools for imaging, measuring and manipulating matter at the nanoscale
- A type of scanning probe microscopy(SPM)
- SPM: Forms images of surfaces using a physical probe that scans the specimen
- Scanning Tunneling microscope(STM, the predecessor of AFM) is also a type of SPM.

HISTORY

- Scanning Tunneling Microscope (STM)
- Developed in 1982 by Binning, Rohrer, Gerber, and Weibel at IBM in Zurich, Switzerland.
- Binning and Rohrer won the Nobel Prize in Physics for this invention in 1986.
- Atomic Force Microscope (AFM)
- Developed in 1986 by Binning, Quate, and Gerber as a collaboration between IBM and Stanford University.
- **Definitions**
- **Scanning Probe Microscopy (SPM):** Consists of a family of microscopy forms where a sharp probe is scanned across a surface
- The two primary forms of SPM consist of:
- **Scanning Tunneling Microscopy (STM)**
- **Atomic Force Microscopy (AFM)** (also called Scanning Force Microscopy (SFM))
- There are 3 primary modes of AFM:
- Contact Mode AFM
- Non-contact Mode AFM
- TappingMode™ AFM

HOW DOES AFM WORKS?

- The information is gathered by "feeling" the surface with a mechanical probe.
- A cantilever with a sharp tip, which is typically silicon or silicon nitride.
- The tip radius of curvature is very small, on the order of nanometers to ensure accuracy.



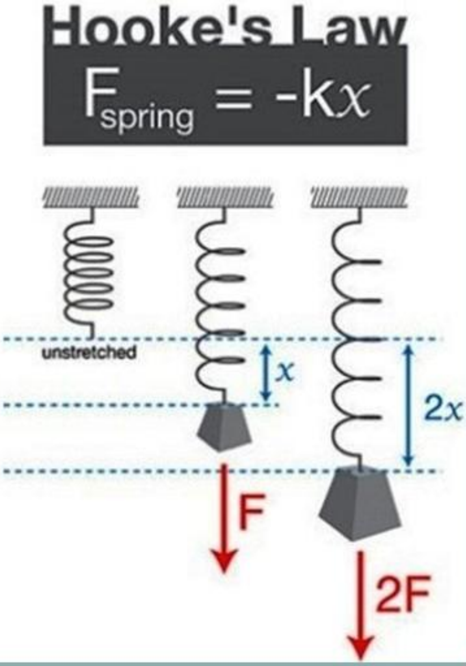
Let's Review Hooke's law

Hooke's law:

The force F needed to extend or compress a spring by some distance x is proportional to that distance.

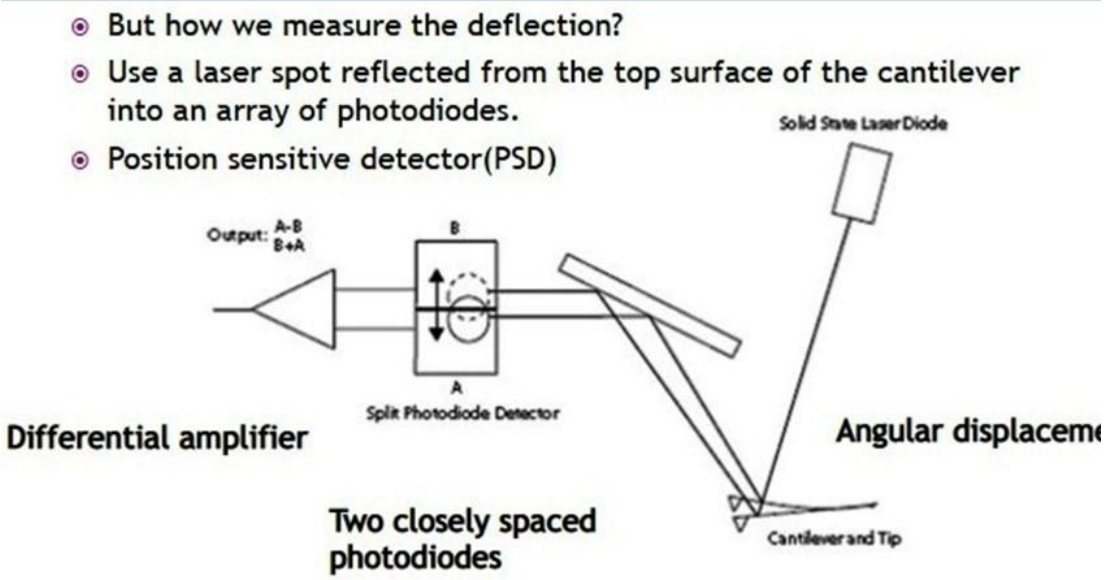
Formula:

F=-kx

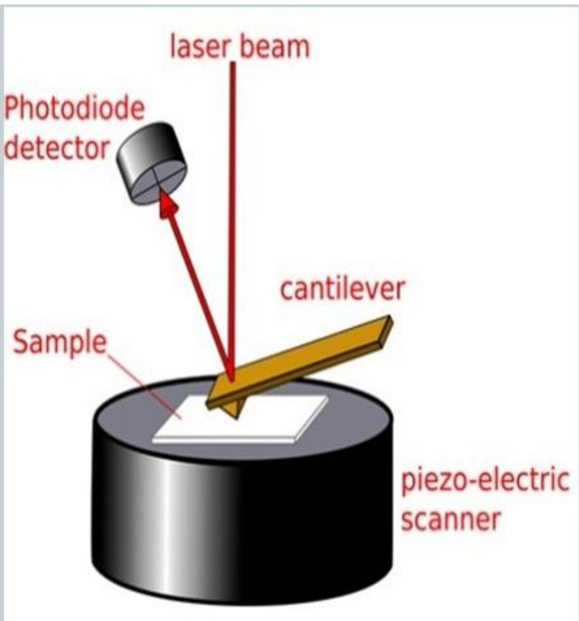


- In atomic scale it is not spring-mass system anymore (contact force, van der Waals force, capillary force, chemical bonding, electrostatic force, magnetic force, etc.).

- So when the tip approaches the surface it can “feel” these force and deflection is measured to be converted into image information.

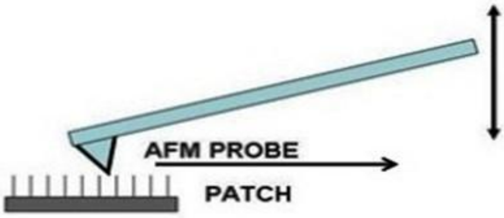


The AFM is a very high resolution type of scanning probe microscopy, with demonstrated resolution of fractions of a nanometer, more than 1000 times better than the optical diffraction limit.

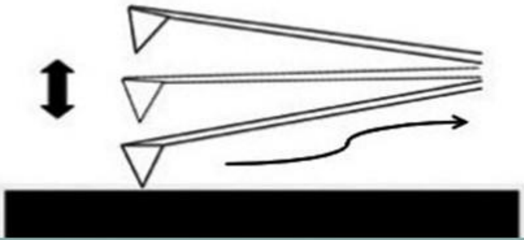


DIFFERENT IMAGING MODES

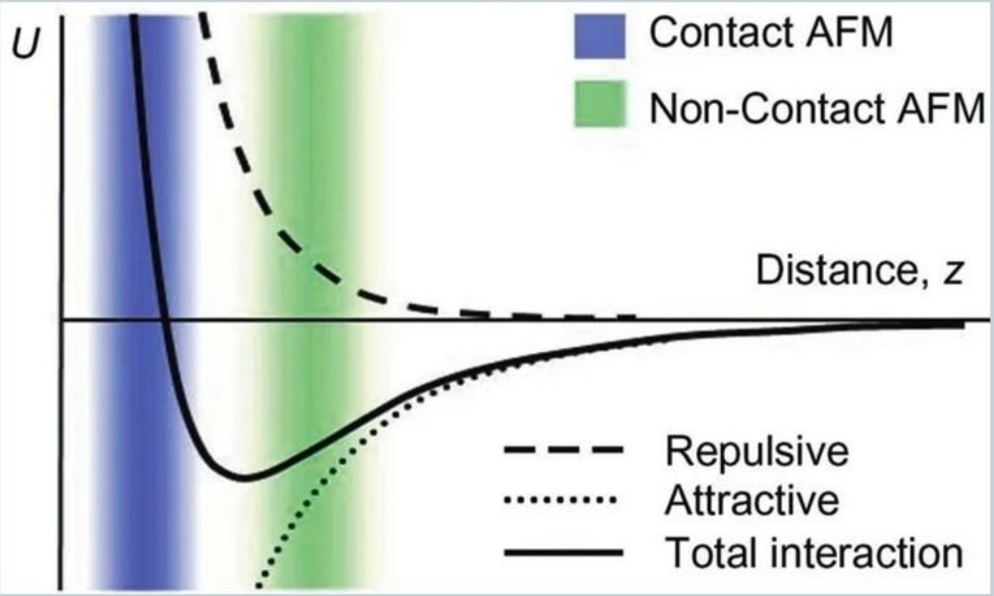
- Static mode (contact mode)
  dynamic mode (non-contact mode and tapping mode)
- Static mode:



- Dynamic mode:



DIFFERENT IMAGING MODES



Van der Waals forces

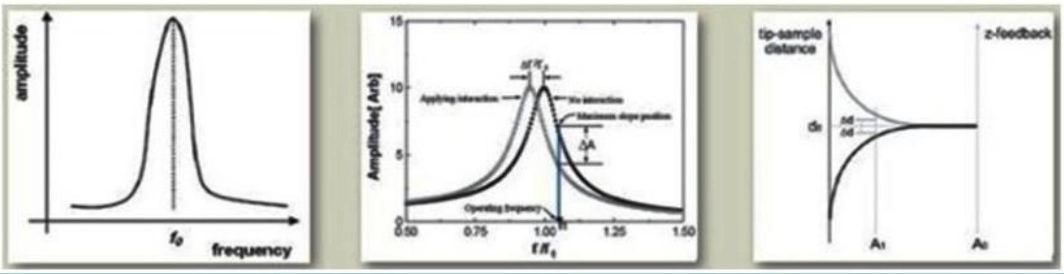
CONTACT MODE

- Attractive forces can be quite strong, cause the tip to contact the surface.
- Mainly used to image hard surfaces when the presence of lateral forces is not expected to modify the morphological features.
- On crystalline surfaces such as mica, Au (111), salt crystals, etc.
- Prone to noise and drift
- low stiffness cantilevers are used to boost the deflection.
- Si probes are more common

CdF_2 films grown on a CaF_2 (111) substrate. Scan is taken in contact mode using a CSC21 probe (now upgraded to HQ:XSC11). Scan size 2 x 2 µm, height 2 nm..

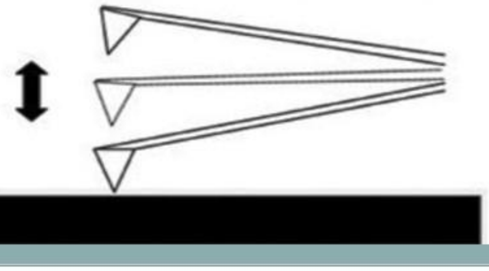
NON-CONTACT MODE

- The tip of the cantilever does not contact the sample surface.
- Oscillated at either its resonant frequency (frequency modulation) or just above (amplitude modulation)
- The van der Waals forces or any other long range force acts to decrease the resonance frequency of the cantilever.
- Maintains a constant oscillation amplitude or frequency by adjusting the average tip-to-sample distance.
- Tip-to-sample distance at each (x,y) data point , construct a topographic image of the sample surface.



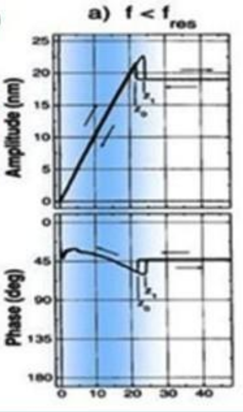
NON-CONTACT MODE

- Does not suffer from tip or sample degradation effects
- If a few monolayers of adsorbed fluid are lying on the surface of a rigid sample, the images may look quite different.
- Frequency modulation: changes in the oscillation frequency provide information about tip-sample interactions.
- Amplitude modulation: changes in the oscillation amplitude or phase provide the feedback signal for imaging



TAPPING MODE

- In ambient conditions, most samples develop a liquid meniscus layer -> keep the probe tip close enough to the sample for short-range forces to become detectable while preventing the tip from sticking to the surface
- The cantilever is driven to oscillate up and down near its resonance frequency. Images are produced by imaging the force of intermittent contacts.
- Lessens the damage done to the surface and the tip.



(b)

"The motivation for this tapping mode AFM is to overcome the difficulty of operating the non-contact mode AFM...
The oscillation amplitude, and therefore, the energy associated with the oscillation, is made to be sufficient to overcome the stickiness of the surface."

Q. Zhong, D. Innis, K., Kjoller, V.B. Elings, Surface Science Letters 290, L688 (1993)

Contact vs Tapping Mode

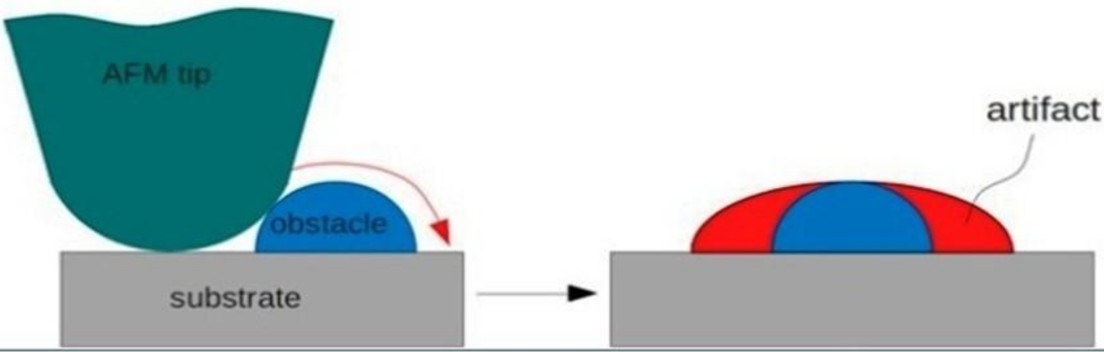
ADVANTAGES	DISADVANTAGES
Contact Mode AFM	
High scan speeds	Lateral (shear) forces can distort features in the image
The only AFM technique which can obtain "atomic resolution" images	The forces normal to the tip-sample interaction can be high
Rough probes with extreme changes in vertical topography can sometimes be scanned more easily.	
Tapping Mode AFM	
Higher resolution on most samples (1 to 5 nm)	Slower scan speed
Lower forces and less damage to soft samples imaged in air	

Advantages & Disadvantages

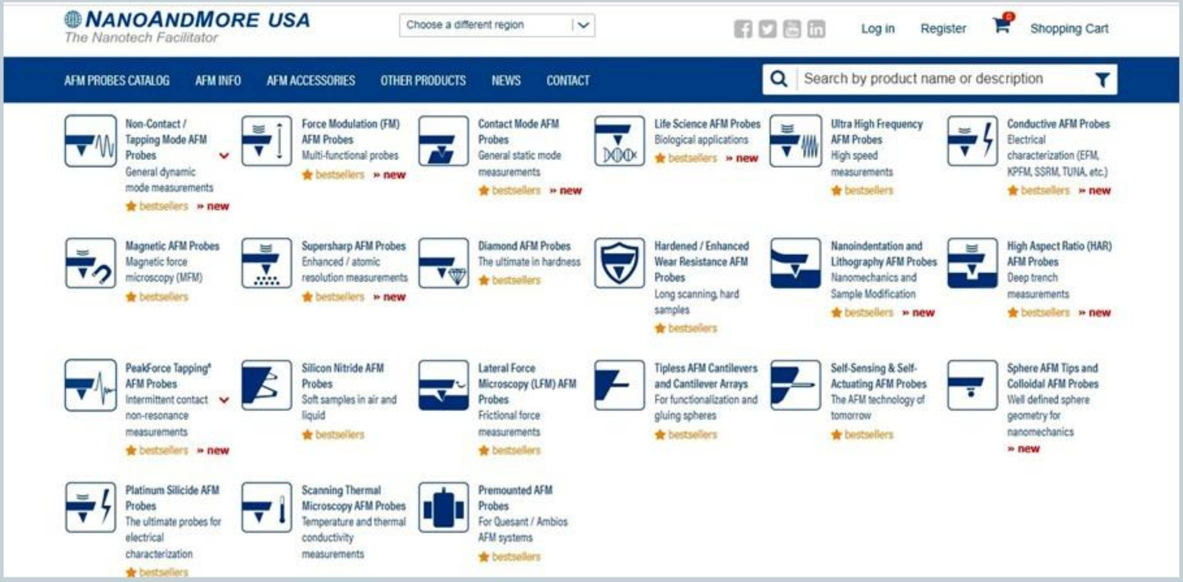
- Three-dimensional surface profile.
- Do not require any special treatments (such as metal/carbon coatings) that would irreversibly change or damage the sample,
- Does not typically suffer from charging artifacts in the final image.
- Can work perfectly well in ambient air or even a liquid environment.
- Higher resolution than SEM, comparable in resolution to STM and TEM.
- Can be combined with a variety of optical microscopy techniques.

Advantages & Disadvantages

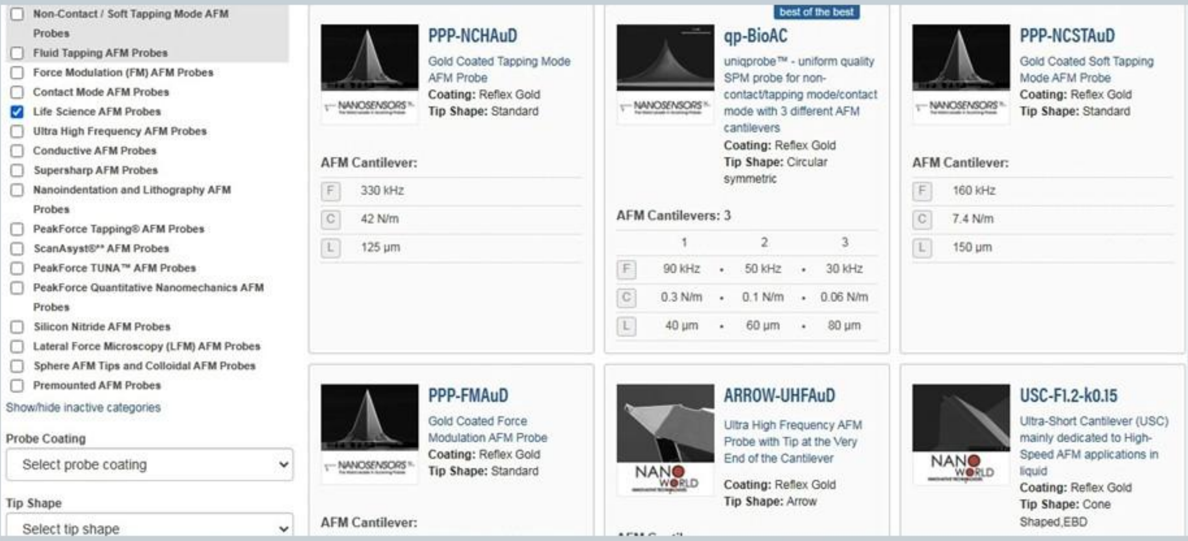
- Single scan image size.
- The scanning speed of an AFM is also a limitation.
- Can be affected by nonlinearity, hysteresis, and creep of the piezoelectric material .
- The possibility of image artifacts, which could be induced by an unsuitable tip, a poor operating environment, or even by the sample itself.
- Cannot normally measure steep walls or overhangs.



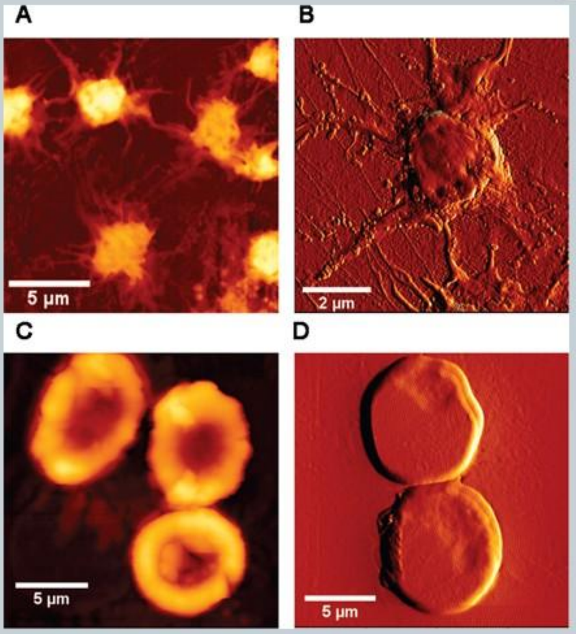
TIPS



TIPS

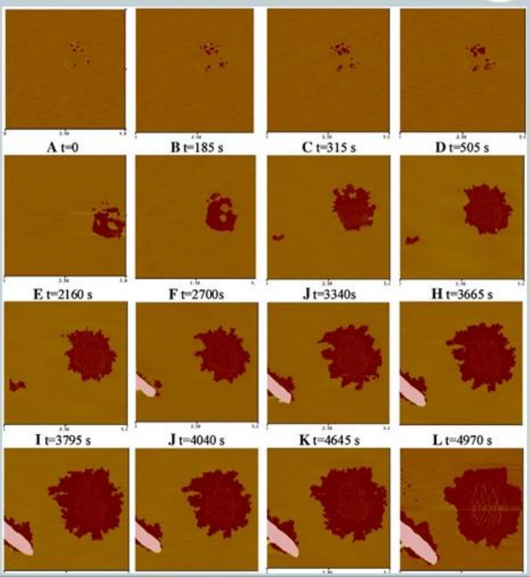


AFM for Life Science



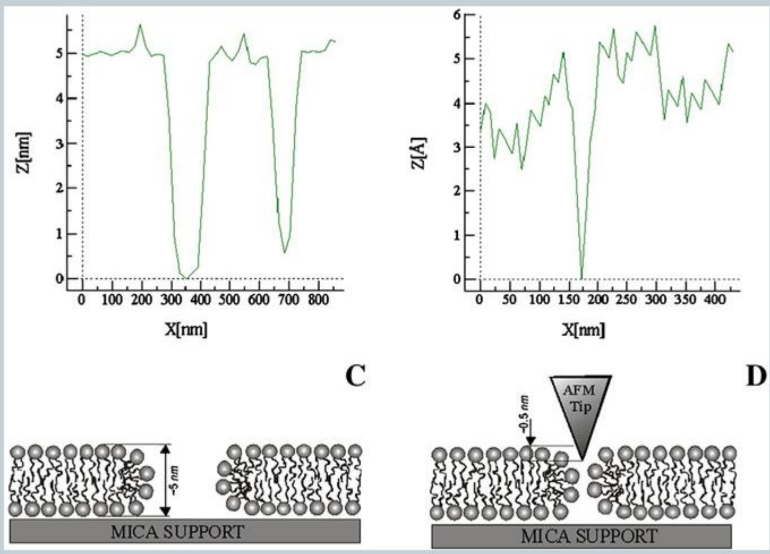
Air tapping-mode AFM images of human platelets (A,B) and human erythrocytes (C,D) from healthy donors, deposited on poly-L-lysine coated glass slides. (A,C) Height images; (B,D) error signal images. AFM images of typical circular, biconcave erythrocytes and spiky human blood platelets.

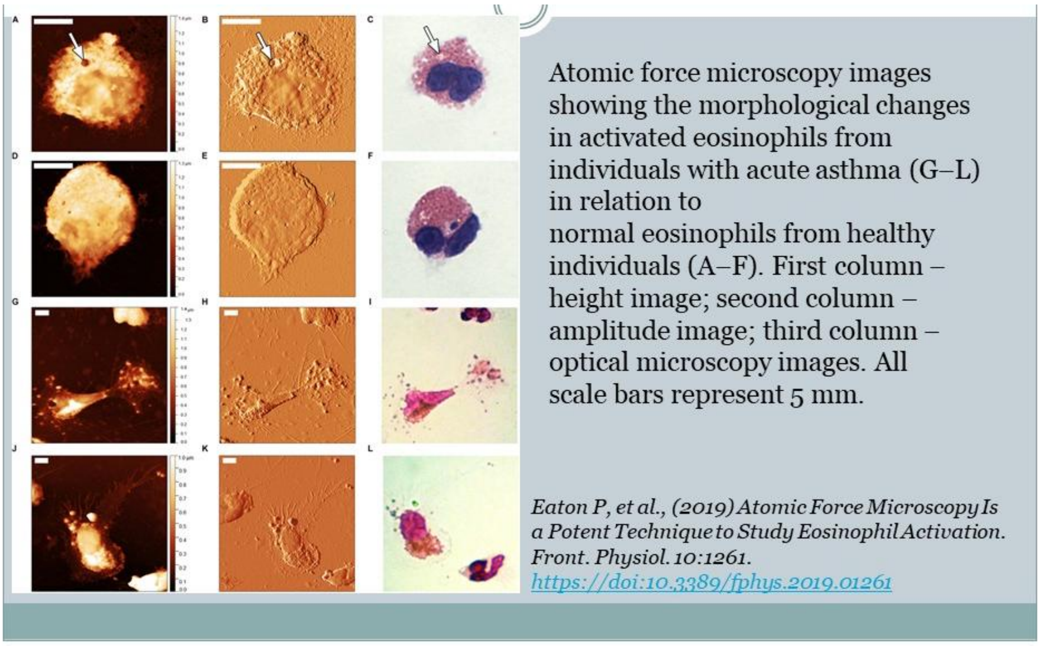
Carvalho, F. A., et al. (2010 ACS nano, 4(8), 4609–4620. https://doi.org/10.1021/nn1009648



A typical time-course set of images from an experiment in which 100 nM of vipoxin's PLA2 solution was injected into the liquid cell (scan size=5×5 μm2). Image A is prior to the enzyme injection. The few bilayers defects (darker areas) are easily distinguishable. Image B is captured 185 s. after enzyme injection. The enlargement of the existing defects is
necked eye detectable. Images C–P are taken in the time interval 315–6340 s after enzyme injection showing occurrence of progressive changes in surface topology of the bilayer as a result of the enzyme action. The darker areas represent structural defects in the lipid bilayer. After enzyme injection, the initial structural defect expands as the top lipid layer is hydrolyzed and the bottom layer spontaneously desorbs

K. Balashev et al. / Biochimica et Biophysica Acta 1808 (2011) 191–198. https://doi:10.1016/j.bbamem.2010.10.008





ATOMIC FORCE MICROSCOPY QUIZ

1. What is AFM?
   - A. A type of electron microscope that produces images of a sample by scanning the surface with a focused beam of electrons
   - B. An optical imaging technique for increasing optical resolution and contrast of a micrograph by means of using a spatial pinhole to block out-of-focus light in image formation
   - C. A technique that uses a very sharp metal wire tip over a surface by applying an electrical voltage to the tip or sample
   - D. A technique that uses a sharp tip to scan a sample, mapping the contours of its surface to record images typically at atomic scale
   - E. A technique that collects and processes information across the electromagnetic spectrum to obtain the spectrum for each pixel in an image

2. Necessary thing for AFM works is
   - A. Video Camera
   - B. Cantilever
   - C. Oculars
   - D. Fluorescent Lamp
   - E. Oil

3. AFM images can be analyzed with
   - A. Rudolf
   - B. Adolf
   - C. Python
   - D. Gwyddion
   - E. ImageJ

4. Image quality depends on

A. Tip quality
B. Silence
C. Vibration
D. Electromagnetic noise
E. All answers are correct

5. AFM can be used to measure
A. Arterial pressure
B. Light speed
C. Temperature
D. Van Der Waals forces
E. Discharge power

6. Which of the following is true about the AFM contact mode
A. Physical contact between the tip and the surface of the sample, high scan speeds, high resolution, no damage to the sample
B. Physical contact between the tip and the surface of the sample, high scan speeds, low resolution, damage to the sample
C. Physical contact between the tip and the surface of the sample, slow scan speeds, low resolution, damage to sample
D. Physical contact between the tip and the surface of the sample, slow scan speeds, high resolution, damage to the sample
E. None of these choices are correct

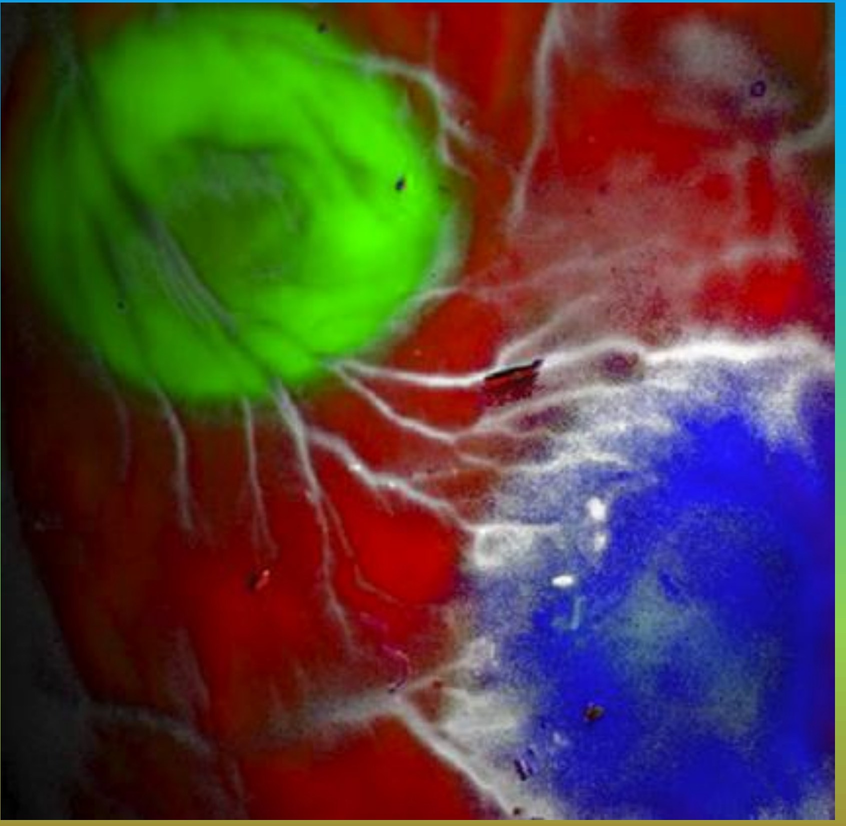
7. For the normal operation of the AFM is necessary
A. Absolute silence
B. Total darkness
C. 60x zoom ocular
D. Scanning in a high-humidity environment
E. No special conditions are needed

8. During scanning, the movement of the cantilever is provided by the
A. Operator
B. Laser
C. Rotor
D. Stator
E. Piezoceramics

9.Function “Gain” needed in order to
A. Avoid breaking the tip
B. Focus the image to the desired height
C. Compensate for touch errors
D. Normalization of the influence of artifacts on the quality of the picture
E. All answers are correct

10. For the operation of the microscope, the sequence of the laser passage should be as follows
A. Mirror–Photodetector–Tip
B. Mirror–Tip–Photodetector
C. Photodetector–Mirror–Tip–Surface
D. Photodetector–Tip–Surface–Mirror
E. Photodetector–Mirror–Tip

HYPERSPECTRAL IMAGING



INTRODUCTION

Hyperspectral imaging (HSI) is a powerful new technology based on spectroscopy. It is based on collecting hundreds of images at different wavelengths from the same spatial area. The collected data form a hyperspectral cube, in which two dimensions represent the spatial coordinates (x,y), while the third coordinate shows wavelength (L). Hyperspectral imaging yields the spectrum for each pixel in the image, making it possible to find objects, identify materials, or detect hidden text or images otherwise invisible to a naked eye using a variety of mathematical algorithms. Additional information about the basics of hyperspectral imaging can be found on wikipedia page or by watching this youtube video.

Nuance EX and FX HSI systems from PerkinElmer are capable of capturing wavelengths between 460-950 nm and 420-720nm respectively at a spatial resolution of 1392×1040 pixels. Nuance custom software can then perform spectral unmixing based on regions-of-interest, real component analysis or spectral libraries derived previously. The camera unit can be mounted on a microscope to analyze the smallest objects. It can also be equipped with long-distance objective for acquiring data from larger objects such as inscriptions on the buildings. For more info about Nuance EX download its PDF manual.

At its core, HSI works by dividing the light spectrum into numerous narrow bands, each representing a specific wavelength of light. These bands range from the visible spectrum (what we can see) to the infrared and ultraviolet regions, which are invisible to the naked eye. By capturing the reflectance or emission of light from an object at each of these wavelengths, HSI generates a comprehensive data set that can be analyzed to identify the unique spectral properties of materials, chemicals, or biological tissues.

One of the most significant advantages of hyperspectral imaging is its ability to detect subtle differences in materials that would otherwise appear identical in standard images. For example, in agriculture, HSI can be used to monitor plant health by detecting changes in chlorophyll content, water stress, or the presence of diseases that are invisible to conventional cameras. In environmental monitoring, it can help identify pollutants or track changes in land use by analyzing the spectral signatures of different soil types or vegetation. In medicine, HSI is being explored for its potential to detect early-stage cancers or monitor wound healing by distinguishing between healthy and diseased tissues based on their spectral characteristics.

HSI systems typically consist of a light source, a dispersive element (such as a prism or diffraction grating), and a detector that records the spectral information. The captured data is often represented as a "hypercube," where two dimensions correspond to spatial coordinates (like a regular image), and the third dimension represents the spectral information. This hypercube contains a wealth of data that can be processed and analyzed using specialized software to extract valuable insights.

While hyperspectral imaging offers remarkable benefits, it also comes with challenges. The vast amount of data generated by HSI systems requires significant storage and computational resources for processing and analysis. Additionally, the equipment needed for hyperspectral imaging is often complex and expensive, which can limit its accessibility for some applications. However, advancements in technology are gradually making HSI more affordable and easier to use, expanding its potential in research and industry.

Ներածություն

Հիպերսպեկտրալ պատկերումը (Hyperspectral Imaging or HSI) սպեկտրոսկոպիայի վրա հիմնված նորագույն և հզոր տեխնոլոգիա է: Այն հիմնված է նույն տարածական հատվածից տարբեր ալիքային երկարություններով հաջորդական պատկերների հավաքագրման վրա: Հավաքված տվյալները ձևավորում են հիպերսպեկտրալ խորանարդ (cube), որտեղ երկու չափերը համապատասխանում են տարածական կոորդինատներին (x, y), իսկ երրորդը՝ ալիքի երկարությանը (λ): HSI թույլ է տալիս ստանալ յուրաքանչյուր պիքսելի սպեկտրալ բնութագիրը, ինչը հնարավորություն է տալիս հայտնաբերել օբյեկտներ, նույնականացնել նյութեր կամ բացահայտել թաքնված գրություններ և պատկերներ, որոնք անզեն աչքով տեսանելի չեն: Այս գործընթացում օգտագործվում են տարբեր մաթեմատիկական ալգորիթմներ և հաշվարկային մեթոդներ՝ տվյալների վերլուծության և դասակարգման համար: HSI հիմունքների վերաբերյալ հավելյալ տեղեկատվություն կարելի է գտնել Վիքիպեդիայի համապատասխան էջում կամ դիտել բացատրական տեսանյութ՝ YouTube հարթակում:

PerkinElmer-ի Nuance EX և FX HSI համակարգերը կարող են որսալ համապատասխանաբար 460-950 նմ և 420-720 նմ ալիքի երկարություններ՝ 1392×1040 պիքսել տարածական լուծաչափով: Համակարգի Nuance հատուկ ծրագրային ապահովումը կարող է իրականացնել սպեկտրալ շերտազատման վերլուծություն՝ հիմնվելով հետաքրքրության գոտիների վրա իրական բաղադրիչների վերլուծության կամ նախկինում ստեղծված սպեկտրալ գրադարանների վրա: Տեսախցիկային միավորը կարելի է տեղադրել միկրոսկոպի վրա՝ մանրագույն օբյեկտների վերլուծության համար: Այն կարող է համալրվել նաև երկարֆոկուս օբյեկտիվով՝ թույլ տալով տվյալների ստացում մեծ օբյեկտներից, օրինակ՝ շինությունների մակագրություններից: Լրացուցիչ տեղեկությունների համար Nuance EX-ի մասին կարելի է ներբեռնել դրա PDF ձեռնարկը:

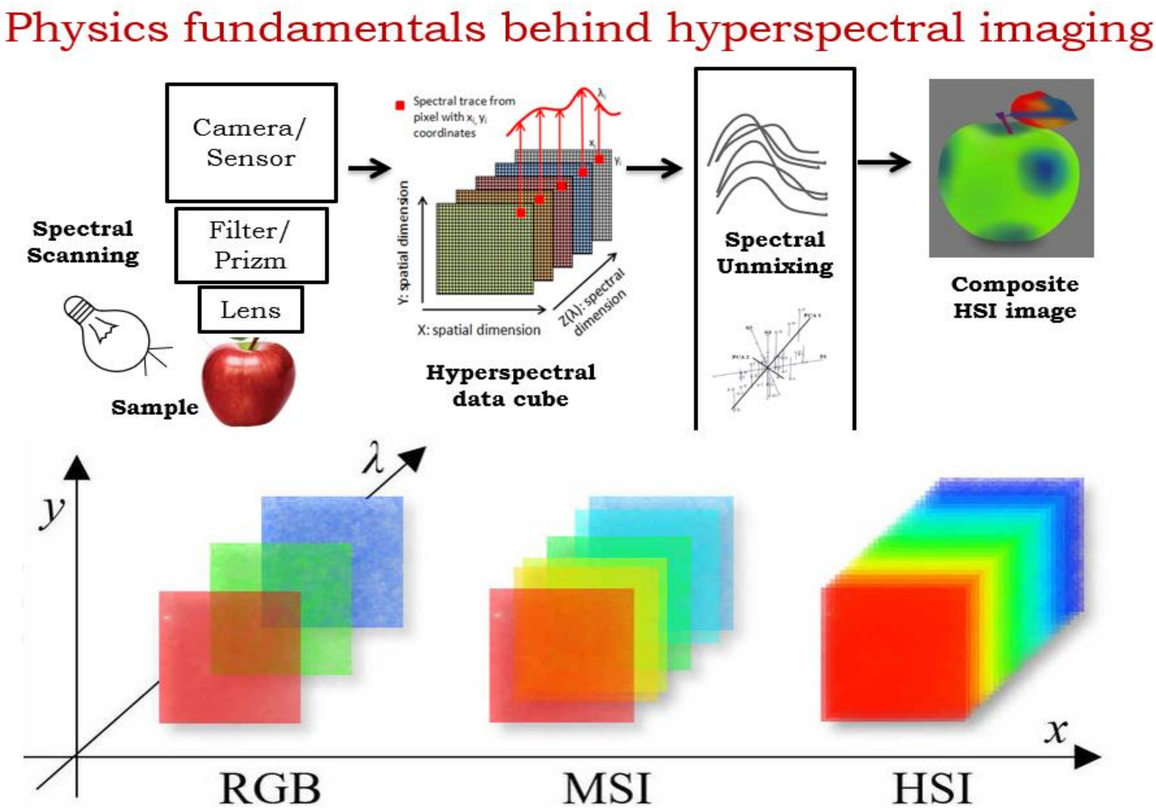
Իր էությամբ HSI գործում է՝ բաժանելով լույսի սպեկտրն բազմաթիվ նեղ գոտիների, որոնցից յուրաքանչյուրը համապատասխանում է լույսի կոնկրետ ալիքային երկարությանը: Այս գոտիները ընդգրկում են ինչպես տեսանելի սպեկտրի հատվածը (այն, ինչ մենք կարող ենք տեսնել), այնպես էլ ինֆրակարմիր և ուլտրամանուշակագույն տիրույթները, որոնք անզեն աչքով տեսանելի չեն: Օբյեկտից արտացոլված կամ արտանետված լույսը գրանցելով յուրաքանչյուր այս ալիքային երկարության համար՝ HSI համակարգը ստեղծում է համալրված տվյալների հավաքածու, որը կարելի է վերլուծել՝ բացահայտելու նյութերի, քիմիական միացությունների կամ կենսաբանական հյուսվածքների եզակի սպեկտրալ հատկությունները:

HSI ամենակարևոր առավելություններից մեկը դրա ունակությունն է հայտնաբերելու նյութերի այն նուրբ տարբերությունները, որոնք սովորական պատկերներում նույնական են թվում: Այս տեխնոլոգիան հնարավորություն է տալիս բացահայտել մանրագույն տարբերություններ՝ հիմնվելով նյութերի սպեկտրալ հատկությունների վրա, ինչը դարձնում է այն անփոխարինելի գործիք տարբեր ոլորտներում: Օրինակ՝ գյուղատնտեսության մեջ HSI կիրառվում է բույսերի առողջության մոնիտորինգի համար՝ հնարավորություն տալով հայտնաբերել քլորոֆիլի պարունակության փոփոխությունները, ջրային սթրեսի (ջրի պակասի) նշանները կամ հիվանդությունների առկայությունը, որոնք սովորական տեսախցիկներով հնարավոր չէ նկատել: Շրջակա միջավայրի մոնիտորինգի ոլորտում այն օգտագործվում է աղտոտիչ նյութերի նույնականացման և հողօգտագործման փոփոխությունների հետևման նպատակով՝ վերլուծելով տարբեր հողային տեսակների և բուսականության սպեկտրալ ստորագրությունները: Բժշկության մեջ HSI լայնորեն ուսումնասիրվում է՝ վաղ փուլերում քաղցկեղի հայտնաբերման, ինչպես նաև վերքերի ապաքինման գործընթացի վերահսկման համար:

HSI համակարգերը սովորաբար բաղկացած են լուսային աղբյուրից, տարալուծող տարրից (օրինակ՝ պրիզմա կամ դիֆրակցիոն ցանց) և դետեկտորից, որը գրանցում է սպեկտրալ տեղեկատվությունը: Ստացված տվյալները սովորաբար ներկայացվում են «հիպերխորանարդի» տեսքով, որտեղ երկու չափերը համապատասխանում են տարածական կոորդինատներին (ինչպես սովորական պատկերում), իսկ երրորդ չափը ներկայացնում է սպեկտրալ տեղեկատվությունը: Այս հիպերխորանարդը պարունակում է մեծ ծավալի տվյալներ, որոնք մշակվում և վերլուծվում են հատուկ ծրագրային ապահովման միջոցով՝ արժեքավոր տեղեկություն ստանալու նպատակով:

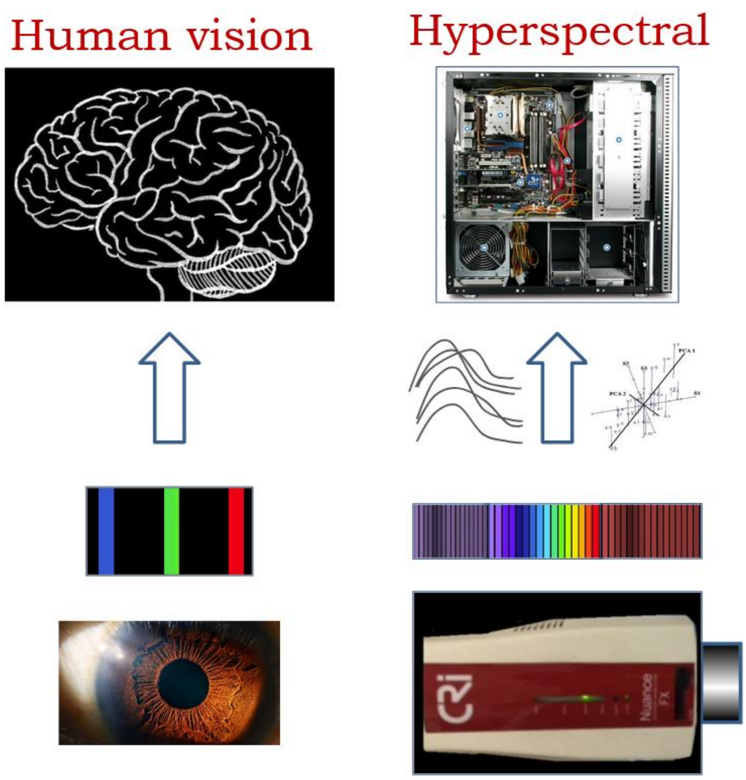
Թեև HSI առաջարկում է նշանակալի առավելություններ, այն ունի նաև մի շարք մարտահրավերներ: HSI համակարգերի կողմից գեներացվող հսկայական տվյալների ծավալը պահանջում է մեծածավալ պահեստային և հաշվարկային ռեսուրսներ՝ տվյալների մշակման և վերլուծության համար: Բացի այդ, HSI համար անհրաժեշտ սարքավորումները հաճախ բարդ և թանկարժեք են, ինչը կարող է սահմանափակել դրանց կիրառելիությունը որոշ ոլորտներում: Այնուամենայնիվ, տեխնոլոգիական առաջընթացը աստիճանաբար դարձնում է HSI-ն ավելի մատչելի և օգտագործման համար հարմար տարբեր գիտական և արդյունաբերական կիրառություններում:

PROTOCOLS AND VISUALS

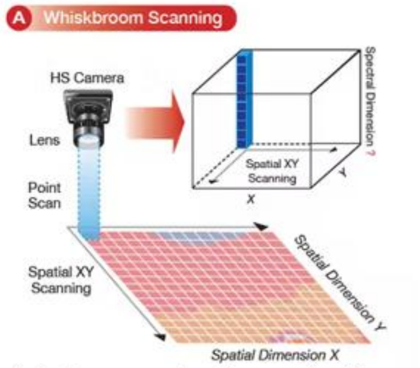


HSI allows to overcome main limitations of human color vision. The limitations are:

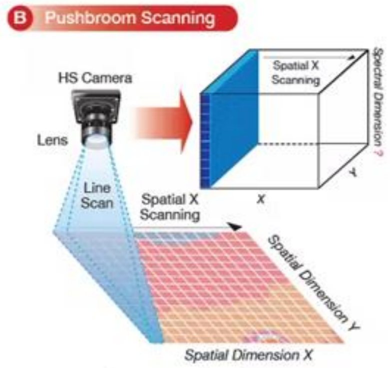
- Subjectivity
- Only few types of photoreceptors
- Limited spectral range
- Needs a lot of light



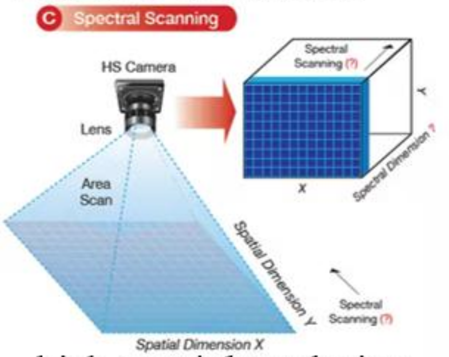
Types of hyperspectral cameras



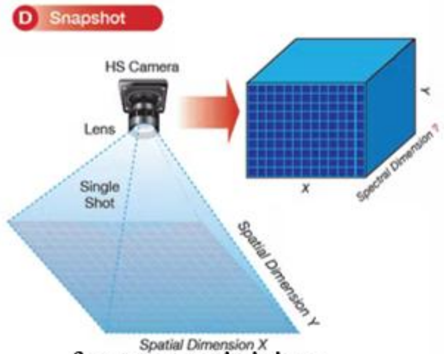
high *spectral* resolution
very slow acquisition



medium xyλ resolution,
mainly suitable for moving targets



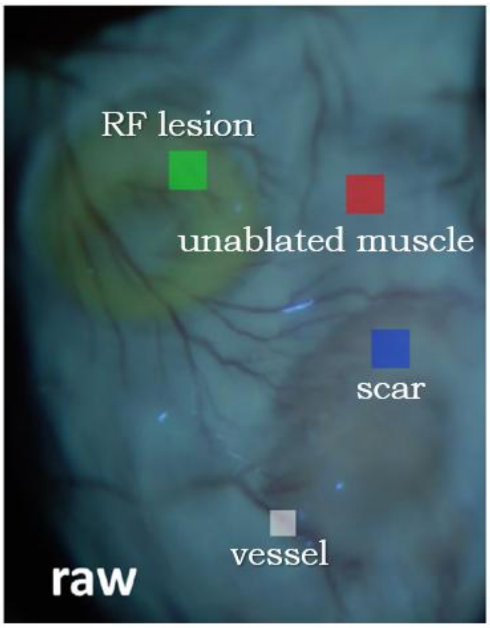
high *spatial* resolution
slow acquisition

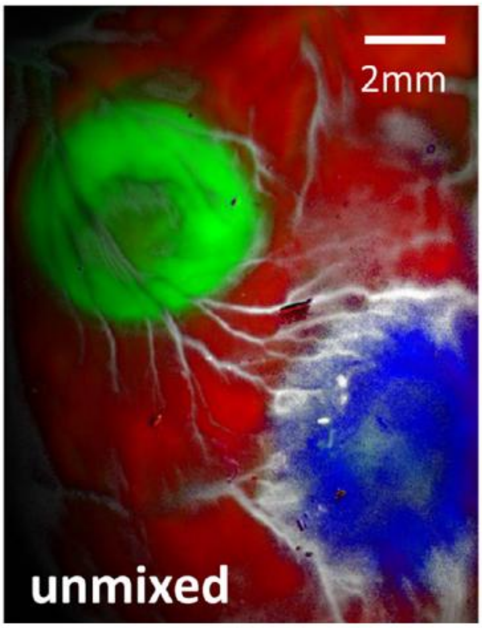


fast acquisition
low xyλ resolution

Halicek et al, Cancers 2019

HSI can help to reveal multiple features or tissue types, e.g. blood vessels, fat, collagen





Swift LM, et al. ***Heart Rhythm*** 2018

Nuance FX and EX HSI systems

Introduction

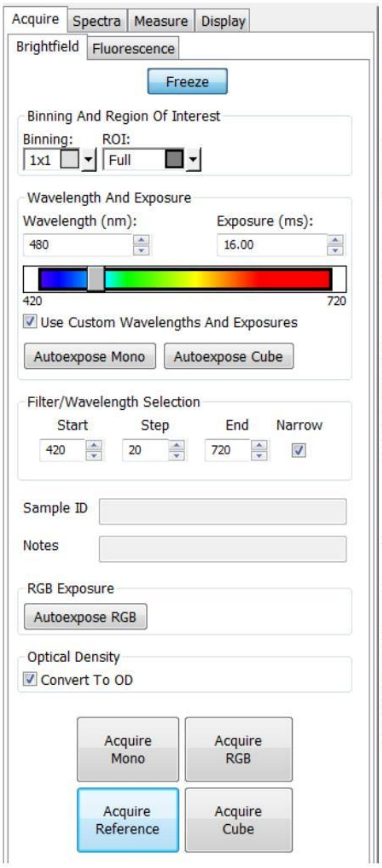
The Nuance Multispectral Imaging System is an advanced imaging platform that facilitates the separation and analysis of overlapping signals in biological tissues. It is designed for use in complex imaging tasks where multiple fluorophores or chromogens are present within a sample. This system is particularly useful in fields like histology, pathology, immunohistochemistry, and molecular biology, where the accurate quantification of multiple markers is critical for understanding cellular processes.

In biological research, the study of complex systems often requires the simultaneous detection of several molecular markers, each associated with different biological targets. Traditional imaging systems often struggle with cross-talk between these signals, especially when markers are co-localized. This cross-talk can significantly reduce image clarity, leading to inaccurate quantification and misinterpretation of results.

The Nuance Multispectral Imaging System addresses these challenges through multispectral imaging and spectral unmixing technologies. By capturing images across a range of wavelengths, the system is able to separate signals based on their unique spectral profiles, even when these signals are overlapping spatially. This results in highly accurate visualization and quantification of each individual marker.

Acquiring Brightfield

1. Freeze / live: Ensure light is diverted to camera and click “live” to display image on screen.
2. Acquire Reference: If converting to OD, move slide to empty field (Empty field should be free of tissue, cells or debris) and acquire reference frame.
3. Autoexpose and Acquire:
   a) Monochrome acquisition
      - Select wavelength of interest
      - Autoexpose Mono
      - Acquire Mono
   b) RGB (Red-Green-Blue) acquisition
      - Autoexpose RGB
      - Acquire RGB
   c) Image cube acquisition
      - Select wavelength range
      - Autoexpose Cube
      - Acquire Cube
4.Binning: Combines multiple pixels into a single pixel (reduces final resolution but increases acquisition speed).
5.Region of Interest: Select all or part of the field to acquire.
6.Sample ID / Notes: Include additional sample notes (acquisition date and parameters are automatically recorded).

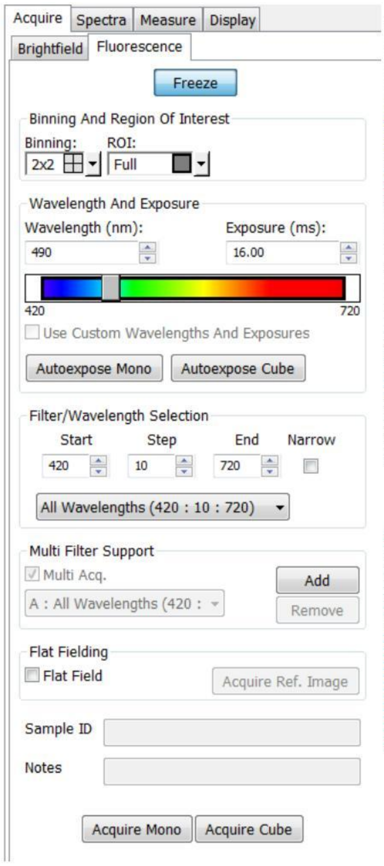


Spectral Libraries

In order to ensure accurate unmixing of component signals from image cubes it is necessary to generate pure spectral libraries from single stained samples. Bring one unstained slide (for fluorescence only). For

BF and fluorescence also bring one single-stained slide for each stain or fluorophore used on experimental samples. When acquiring image cubes for each of these, use the same filter and wavelength selections as will be used for the experimental samples.

Acquire Fluorescence



1. Freeze / live: Ensure light is diverted to camera and click "live" to display image on screen.
2. Acquire Reference: To correct for uneven field illumination, move slide to area of uniform illumination and acquire reference frame. Flat Fielding makes it possible for the Nuance software to create better, more evenly bright mono images and image cubes in fluorescence. If you acquire fluorescence images or cubes without Flat Fielding, you may notice that the outer edges of acquired images are slightly darker than the rest of the image. Before acquiring a mono image or spectral cube, acquire a reference - or background - image. This requires a fluorescence sample slide that is evenly bright across the field of view. Once you have taken a reference image, it will be saved with the current Nuance protocol, if you save the protocol.
3. Autoexpose and Acquire:
   a) Monochrome acquisition
      • Select wavelength of interest • Autoexpose Mono
      • Acquire Mono
   b) Image cube acquisition
      • Select wavelength range
      • Add multiple filters (optional) • Autoexpose Cube
      • Acquire Cube
4. Binning: Combines multiple pixels into a single pixel (reduces final resolution but increases acquisition speed).
5. Region of Interest: Select all or part of the field to acquire.
6. Sample ID / Notes: Include additional sample notes (acquisition date and parameters are automatically recorded).

Manual Compute Spectra

Identifying Mixed Spectra

1. Click Draw then select a region on the image corresponding to the desired signal.
2. Name the selected spectrum (e.g. autofluorescence, DAPI, etc.)

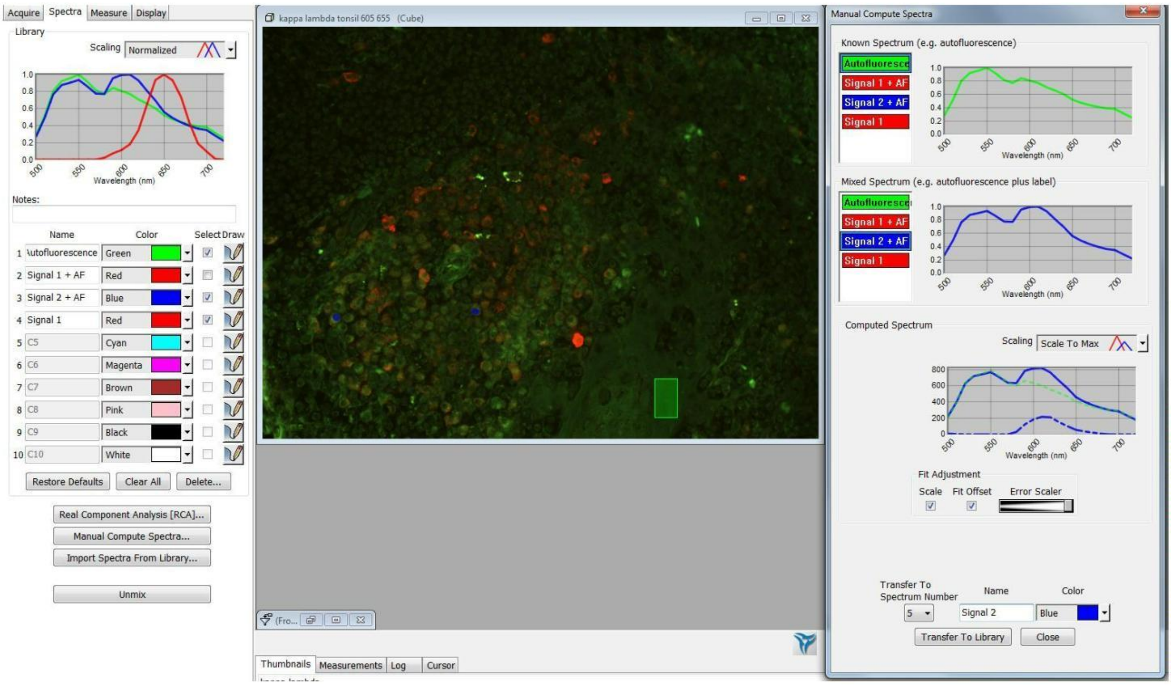
Notes: Repeat steps 1-2 to identify each component signal, selecting a new number, color and label each time. Ideally each mixed spectrum added to the spectral library from single stained samples (autofluorescent signal would come from unstained sample).

Generating Pure Spectral Library

3. Click Manual compute spectra
   a) Select known spectrum Known signal is the autofluorescence generated in steps 1 and 2 from an unstained sample or background region and will be subtracted from the mixed signal.
   b) Select Mixed spectrum: Mixed spectra added to the spectral library during steps 1 and 2 using single stained samples or by carefully selecting regions positive for only one of the fluorescent stains.
   c) Computed Spectrum
   i. Error scaling: set graph as "scale to max" and adjust scale to line up mixed and known spectral curves in regions outside signal area.
   ii. Use "fit offset" to enhance weak signals

d) Transfer to library, repeat until all signals have been separated.

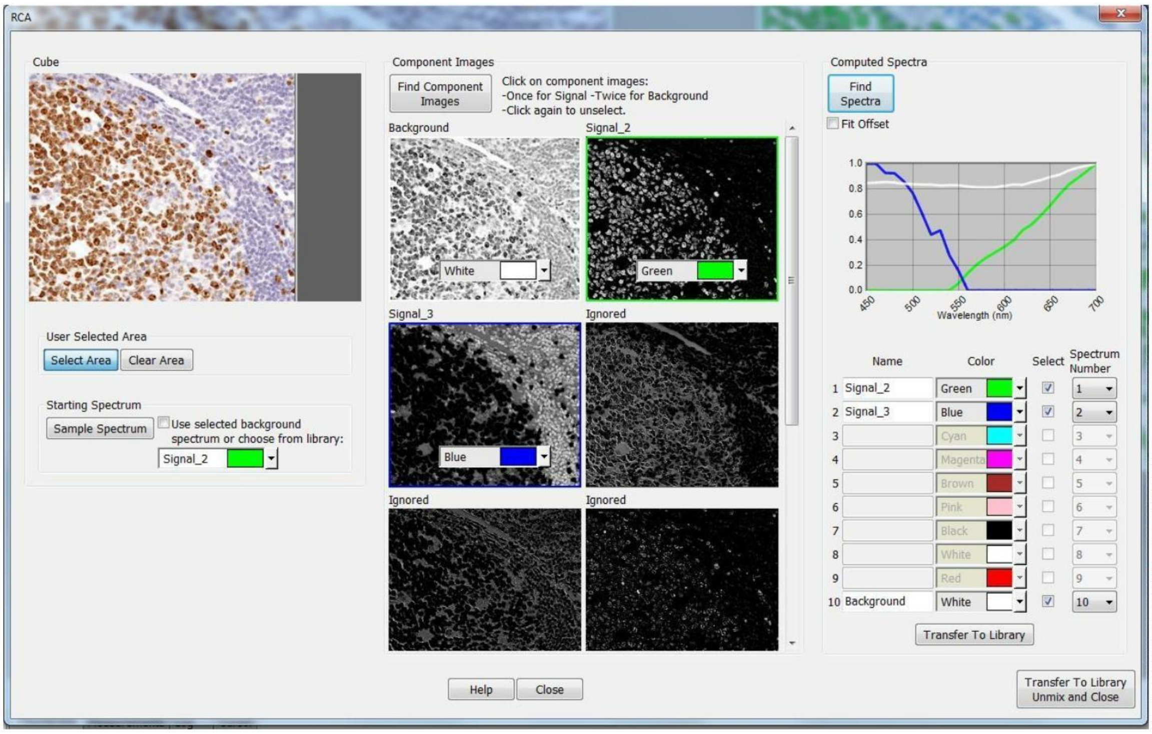
4. Select signals and click Unmix: Once spectral library is generated all image cubes can be unmixed.



Real Component Analysis

After acquisition click Spectra tab and then click Real Component Analysis (RCA) to begin

1. Starting Spectrum (Optional): If background signal has already been added to library (manually or by importing from spectral library), select this signal as starting spectrum.



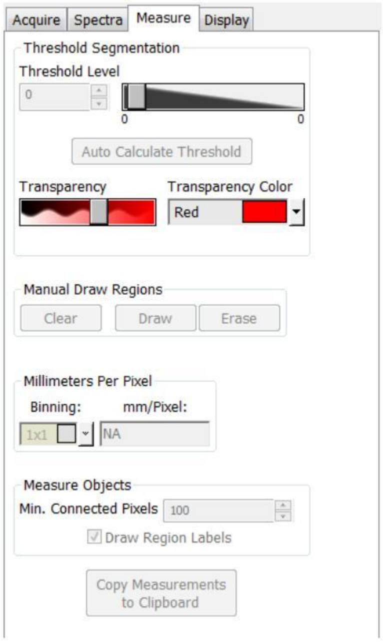
2. Click Find component images: After clicking, find images below with clear signal (white indicates bright signal), then assign label (1 click labels as “Signal_#”, 2 clicks labels as “Background”, 3 clicks resets to “ignored”)

3. Click Find spectra: Click “Fit offset” for weak signals 4. Signal Labels:

a. Select each unique signal (check box)
b. Give each a name (e.g. autofluorescence, DAPI, etc.)
c. Change the color if necessary (this will change how the spectral graphs look but will not change the image appearance)
d. Choose the spectrum number to which you would like to transfer each spectrum.

5. Click Transfer to library, Unmix and Close: This will transfer selected spectra to the indicated spectrum number in your active library (overwriting any spectra currently occupying these slots).

Threshold Segmentation Analysis



1. Threshold level: adjust the slider to set the minimum signal intensity for pixels to be classified as positive.
2. Minimum connected pixels: sets smallest size of positive region of interest (ROI).
3. Manual segmentation: Can draw/copy/move/000e ROIs.
4. Threshold mask: set the color and transparency of the mask used to indicated positive ROIs.
5. Set Scale: Indicated mm/pixels if known (use fiduciary to determine if unknown).

For each ROI the following are reported:

- Avg. signal or Avg. signal/sec
- Total signal or Total signal/sec
- Max signal
- Area (pixels and mm^2) • Major/minor axis
- (x, y) coordinates of ROI center

Data can be copied/pasted/exported to:

- Microsoft Excel • GraphPad Prism
- Other spreadsheet software

Display Control Utility

1. Select the correct Composite Coloring Style. 2. Display adjustments:

a. Select whether to adjust the whole image or single layer.
b. For single layer adjustments check the layer to be adjusted. You can also select which layers are visible here.

3. Scaling:

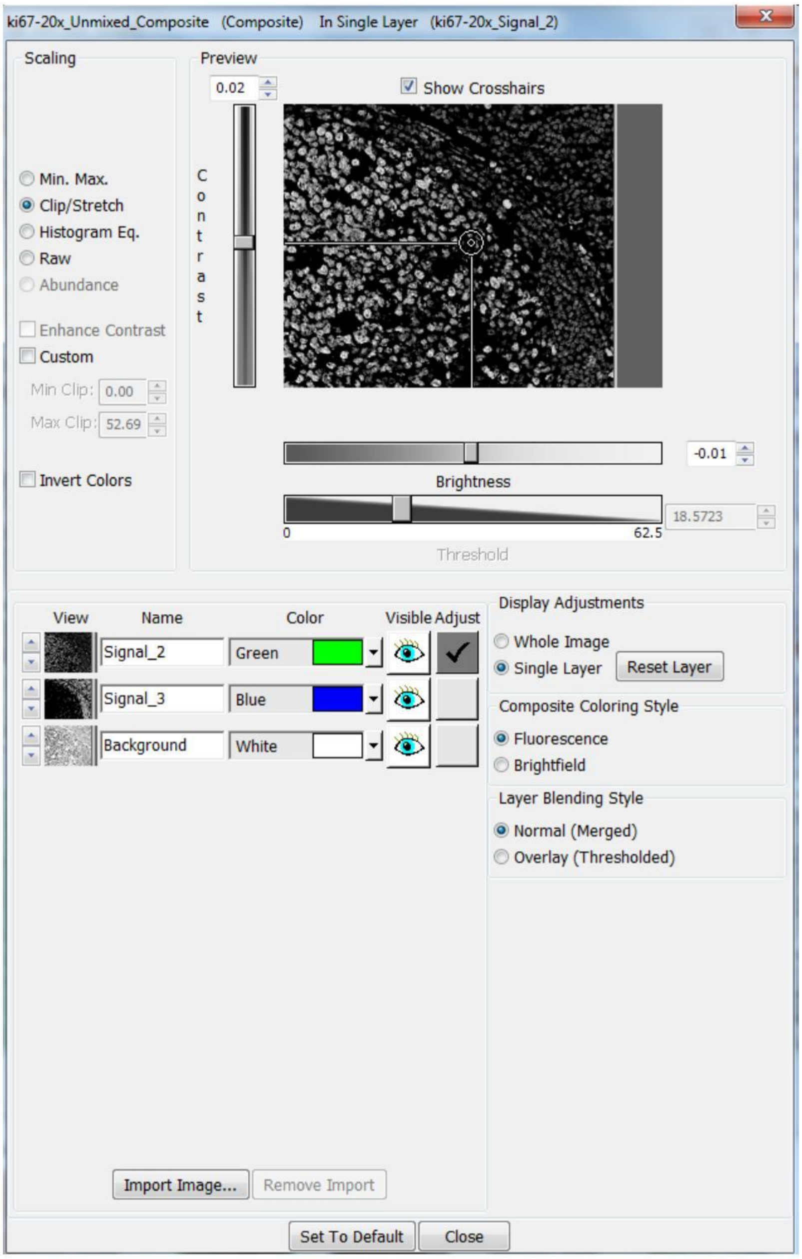
a. Min-Max (minimum pixel intensity - 0; maximum pixel intensity - 255)
b. Clip stretch (0.01% lowest pixel intensity - 0; 0.01% highest pixel intensity - 255)
c. Custom clip stretch (can change % of lowest/highest intensity pixels stretched to 0/255)
d. Abundance scales all pixels to maximum in composite image (preserves relative abundance)

4. Brightness/contrast tool: Drag crosshairs to simultaneously adjust brightness and contrast. Or adjust sliders separately.

For bright field ensure that background color is set to white. By default, layer blending is set to *Merged*, in which component images are mixed based on intensity. To prioritize certain signals, switch to *Overlay* mode. Here component threshold masks are layered on top of each other, and you can choose to highlight a select signal by placing it on top.

Colocalization Analysis

1. Settings: If you have saved previous colocalization setting these can be loaded here. File must contain same # of spectra as the unmixed composite image to be analyzed.
2. ROI selection: Use the ROI draw tools to highlight part of the unmixed composite image for colocalization analysis. By default, the entire image is selected.
3. Thresholding: For each component signal set the minimum signal intensity (Threshold), set the minimum object size (Minimum Pixels), and set the Mask color and Visibility (optional).
4. Colocalization:
    a. Identify colocalization marker(s).
    b. Set denominator/ROI (i.e. full image or region of image).
5. Statistics: Reports % colocalization, pixel counts, and component stats in given ROI.
    a. Export data to Microsoft Excel/GraphPad Prism/Other spreadsheet software.
6. Save Settings for future use.



HYPERSPECTRAL IMAGING QUIZ

1. What is hyperspectral imaging?
   A. A technique used to capture detailed information about the spectral characteristics of objects.
   B. A type of imaging that can see through walls
   C. A tool used to measure the temperature of objects
   D. A technology used to track the movement of objects

2. How does hyperspectral imaging allow for the identification of specific materials or substances?
   A. By detecting the unique chemical composition of different materials
   B. By using x-ray vision to see through objects
   C. By detecting differences in the refractive index of materials
   D. By capturing spectral information from each pixel of an image

3. What are most common current application of hyperspectral imaging in the medical field?
   A. Creating 3D holograms of your internal organs
   B. Creating 3D models of the brain
   C. Identifying cancerous tissue on the surface of the skin
   D. Analyzing the structure of bones and joints

4. What types of light sources are typically used in reflectance-based hyperspectral imaging?
   A. Ultraviolet LEDs
   B. Infrared LEDs
   C. Infrared lasers
   D. Broadband light sources or white LEDs

5. What is the main limitation of using hyperspectral imaging for medical imaging applications?
   A. It is not capable of capturing high-resolution images
   B. It requires highly specialized technical skills to implement
   C. It can only be used for surface-level imaging and cannot penetrate deep tissue
   D. It cannot differentiate between healthy and diseased tissue

6. What is auto-fluorescence?
   A. Auto-fluorescence is a process where an organism emits light as a result of a chemical reaction within its body, similar to how fireflies glow in the dark
   B. Auto-fluorescence is a phenomenon in which light is absorbed and then re-emitted by a substance, without the need for external fluorescent dyes
   C. Auto-fluorescence refers to the automatic adjustment of the brightness and contrast in digital imaging
   D. Auto-fluorescence is a term used to describe the natural coloration of animals, which is used for camouflage or attracting mates

7. How might hyperspectral imaging technology evolve or advance in the coming years?
   A. By becoming more compact and affordable
   B. By incorporating artificial intelligence and machine learning techniques
   C. By expanding its applications in fields such as agriculture, forestry, medicine, and mineral exploration
   D. All of the above

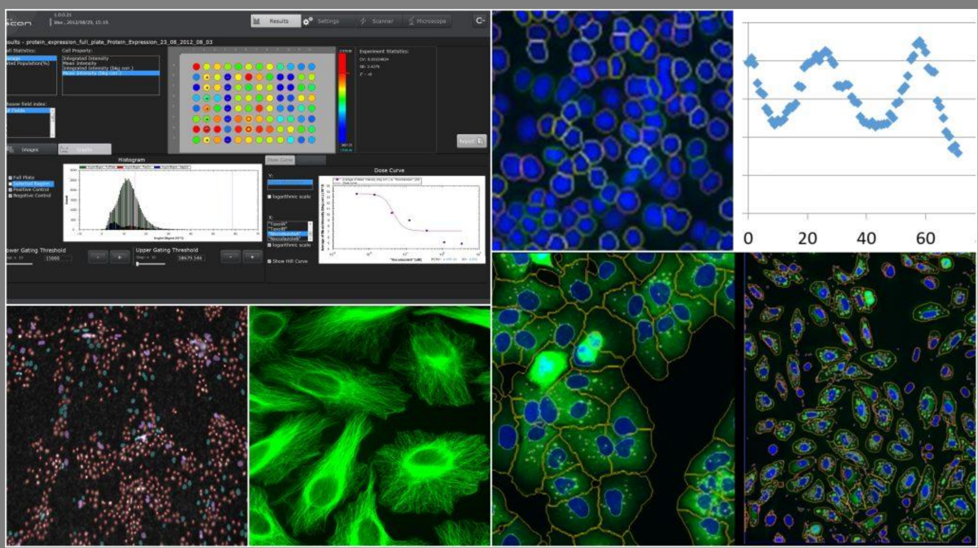
8. How does autofluorescence-based hyperspectral imaging (Auf-HSI) differ from reflectance-based hyperspectral imaging (Ref-HSI)?

A. Auf-HSI requires external light illumination, whereas Ref-HSI does not.
B. Auf-HSI is limited to non-biological samples, whereas Ref-HSI can be used for both biological and non-biological samples.
C. Ref-HSI needs a specific dye, while Auf-HSI does not.
D. Auf-HSI is dependent on intrinsic fluorescence emission from the sample, while Ref-HSI measures light reflected off the sample.

9. What types of materials are suited for hyperspectral imaging?
A. Hyperspectral imaging is exclusively suited for imaging metallic substances due to their high reflectance
B. Only transparent materials can be imaged using hyperspectral imaging because of their light transmission properties
C. Hyperspectral imaging is suitable only for biological tissues as it was specifically developed for medical and biological applications
D. Hyperspectral imaging can be used on a wide range of materials, including plants, minerals, and biological tissues

10. How is hyperspectral imaging (HSI) typically classified based on acquisition mode?
A. Spatial and temporal scanning
B. Spectral and spatial scanning
C. Point and area scanning
D. Band interleaved and band-sequential

IMAGE PROCESSING



INTRODUCTION

Image processing is a fascinating and essential field that involves the manipulation and analysis of images to enhance their quality, extract useful information, or convert them into different forms. It bridges the gap between raw image data captured by devices like cameras or scanners and meaningful visual information that can be used in a wide range of applications, from medical diagnostics and satellite imaging to computer vision and digital art.

At its core, image processing involves applying algorithms and techniques to digital images, which are represented as matrices of pixel values. Each pixel in an image holds information about the intensity of light or color at a specific point, and by manipulating these pixel values, we can alter the appearance of the image or analyze its content.

Image processing tasks can be broadly categorized into image enhancement, image restoration, image segmentation, and image analysis. Image enhancement is one of the most common and straightforward applications of image processing. It involves improving the visual appearance of an image by adjusting factors like brightness, contrast, sharpness, and noise levels. For instance, enhancing an underexposed photograph by increasing its brightness or sharpening a blurry image to bring out fine details are typical examples of image enhancement.

Image restoration focuses on recovering the original image from a degraded version This could involve removing noise, correcting blurriness caused by camera motion, or compensating for distortions introduced during image acquisition. Restoration techniques are crucial in medical imaging, where clear images are vital for accurate diagnosis.

Image segmentation is the process of dividing an image into meaningful regions or objects. This step is often crucial in image analysis, as it allows for the identification and isolation of specific parts of an image. In medical imaging, segmentation can be used to delineate different tissues, organs, or tumors within a scan, enabling more precise analysis.

Image analysis goes beyond simply processing the image and involves extracting meaningful information from it. This could include recognizing patterns, detecting edges, measuring areas or shapes, and even interpreting the content of the image. The field of image processing is heavily reliant on mathematical concepts, particularly in areas like linear algebra, calculus, and statistics. Understanding how to apply these concepts through algorithms allows us to manipulate images effectively. For instance, Fourier transforms are used to analyze the frequency components of images, which can be useful in tasks like image compression or filtering. Image processing is a rapidly evolving field, with advancements in computing power and algorithms driving innovations in artificial intelligence and machine learning. These technologies are increasingly being integrated into image processing tasks, enabling more sophisticated analysis and real-time processing of large image datasets. Two widely used software platforms for image processing in bioimaging and medical research are Fiji (an enhanced version of ImageJ) and CellProfiler.

Image **processing is the class of methods that deal with manipulating images through the use of computer algorithms**. Bioimage analysis can help you to automatically analyze large amounts of image data (such as magnetic resonance imaging (MRI), computed tomography (CT), ultrasound, hyperspectral images, optical microscopy and many more); to reproducibly extract quantitative information from images (to collect quantitative measurements in time and space), to quantify the form and structure of cells and organisms.

Ներածություն

Պատկերների մշակումը գիտության և տեխնոլոգիայի մի հետաքրքրաշարժ ու հիմնարար ոլորտ է, որը ներառում է պատկերների վերամշակման և վերլուծության գործընթացները՝ դրանց որակը բարելավելու, օգտակար տեղեկատվություն ստանալու կամ տվյալները տարբեր ձևաչափերի փոխարկելու նպատակով: Այն ծառայում է որպես կապ չմշակված պատկերային տվյալների և իմաստավորված տեսողական տեղեկատվության միջև՝ ապահովելով անցում այնպիսի համակարգերի համար, ինչպիսիք են տեսախցիկները կամ սկաներները, դեպի կիրառելի տվյալներ: Պատկերների մշակման մեթոդները լայնորեն կիրառվում են տարբեր ոլորտներում՝ սկսած բժշկական ախտորոշումից և արբանյակային պատկերներից մինչև համակարգչային տեսություն և թվային արվեստ:

Իր էությամբ, պատկերների մշակումը հիմնված է թվային պատկերների վրա ալգորիթմների և մեթոդների կիրառման վրա, որոնք ներկայացված են որպես պիքսելային արժեքների մատրիցաներ: Պատկերի յուրաքանչյուր պիքսել պարունակում է տվյալներ տվյալ կետի լուսավորության ինտենսիվության կամ գույնի վերաբերյալ, և պիքսելների արժեքների փոփոխման միջոցով հնարավոր է փոփոխել պատկերի տեսքը կամ վերլուծել դրա բովանդակությունը:

Պատկերների մշակման խնդիրները կարելի է պայմանականորեն բաժանել մի քանի հիմնական կատեգորիաների՝ պատկերի բարելավում, վերականգնում, հատվածավորում և վերլուծություն: **Պատկերի բարելավումը** պատկերների մշակման ամենատարածված և պարզ կիրառություններից է: Այն ներառում է պատկերի տեսանելի որակի բարելավում՝ փոփոխելով պայծառությունը, հակադրությունը, սրությունը կամ աղմուկի մակարդակը: Օրինակ՝ թույլ լուսավորված լուսանկարը պայծառացնելը կամ անուր պատկերը սրացնելը՝ մանրամասները հստակեցնելու համար, պատկերի բարելավման դասական օրինակներ են:

Պատկերի վերականգնումը նպատակ ունի վերականգնել սկզբնական պատկերը՝ աղավաղված տարբերակից: Սա կարող է ներառել աղմուկի հեռացում, անսրության ուղղում (օրինակ՝ տեսախցիկի շարժման հետևանքով), կամ աղավաղումների փոխհատուցում՝ առաջացած պատկերների ստացման ընթացքում: Վերականգնման մեթոդները կարևոր դեր ունեն բժշկական պատկերագրման մեջ, որտեղ հստակ պատկերներն անհրաժեշտ են ճշգրիտ ախտորոշման համար:

Պատկերի հատվածավորումը գործընթաց է, որով պատկերը բաժանվում է իմաստային մասերի կամ առարկաների: Այս քայլը հաճախ առանցքային է պատկերի վերլուծության մեջ, քանի որ թույլ է տալիս նույնականացնել և առանձնացնել պատկերի կոնկրետ հատվածները: Բժշկական պատկերագրման դեպքում հատվածավորումը կարող է կիրառվել տարբեր հյուսվածքներ, օրգաններ կամ ուռուցքներ առանձնացնելու համար, ինչը հնարավորություն է տալիս իրականացնել ավելի ճշգրիտ և նպատակային վերլուծություն:

Պատկերի վերլուծությունը գերազանցում է պատկերների պարզ մշակման սահմանները և ներառում է դրանցից իմաստավորված տեղեկատվության արդյունահանումը: Սա կարող է ներառել նախշերի ճանաչում, եզրերի հայտնաբերում, մակերեսների կամ ձևերի չափում, ինչպես նաև պատկերի բովանդակության իմաստային մեկնաբանություն: Պատկերների մշակման ոլորտը հիմնված է մաթեմատիկական գիտելիքների վրա՝ հատկապես գծային ալգեբրայի, հաշվարկի և վիճակագրության ոլորտներում: Այս հասկացությունները ալգորիթմների միջոցով կիրառելու ունակությունը հնարավորություն է տալիս արդյունավետ կերպով կառավարել և փոխակերպել պատկերները: Օրինակ՝ Ֆուրիեի փոխակերպումները կիրառվում են պատկերների հաճախականային բաղադրիչները վերլուծելու համար, ինչը կարևոր է պատկերի սեղմման կամ ֆիլտրացման խնդիրներում: Պատկերների մշակման ոլորտը արագ զարգացող ճյուղ է. հաշվողական հզորության և ալգորիթմների զարգացման շնորհիվ այն մեծապես ինտեգրվում է արհեստական բանականության և մեքենայական ուսուցման տեխնոլոգիաների հետ: Այս մոտեցումները հնարավորություն են տալիս իրականացնել ավելի բարդ վերլուծություն և իրական ժամանակում մշակել մեծածավալ պատկերային տվյալների հավաքածուներ: Կենսապատկերագրման և բժշկական հետազոտությունների ոլորտում լայնորեն կիրառվող երկու ծրագրային հարթակ են **Fiji**-ն (ImageJ-ի ընդլայնված տարբերակը) և **CellProfiler**-ը:

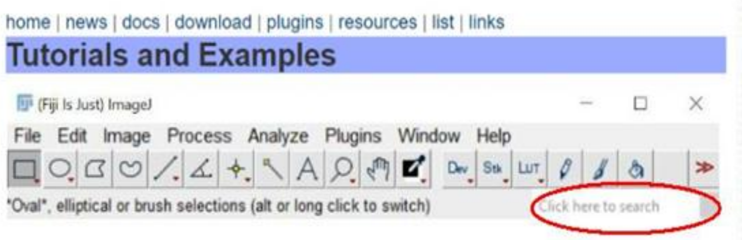
Պատկերների մշակումը մեթոդների այն դասն է, որը զբաղվում է պատկերների մշակմամբ և փոփոխմամբ՝ համակարգչային ալգորիթմների կիրառման միջոցով: Կենսապատկերների վերլուծությունը (bioimage analysis) հնարավորություն է տալիս ավտոմատ կերպով վերլուծել մեծածավալ պատկերային տվյալներ՝ օրինակ մագնիսառեզոնանսային տոմոգրաֆիայի (MRI), համակարգչային տոմոգրաֆիայի (CT), ուլտրաձայնային պատկերների, հիպերսպեկտրալ պատկերների, օպտիկական միկրոսկոպիայի և այլ աղբյուրներից: Այն հնարավորություն է տալիս ստանալ քանակական տեղեկատվություն պատկերներից՝ ժամանակի և տարածության առումով կրկնելի ձևով, ինչպես նաև քանակական գնահատել բջիջների և օրգանիզմների ձևն ու կառուցվածքը:

PROTOCOLS AND VISUALS

ImageJ is open source software for processing and analyzing scientific images. ImageJ facilitates image analysis techniques including image processing, colocalization, deconvolution, registration, segmentation, tracking, visualization, and more. fiji is an image processing package – a "batteries-included" distribution of ImageJ, bundling many plugins which facilitate scientific image analysis.

Beginning

Use Help! Most of the problems have already solutions. FIJI contains a vast amount of plugins. The *Documentation* option has a number of tutorials that guide you through a variety of typical tasks. Search box with keywords to find the steps to solve your issue.

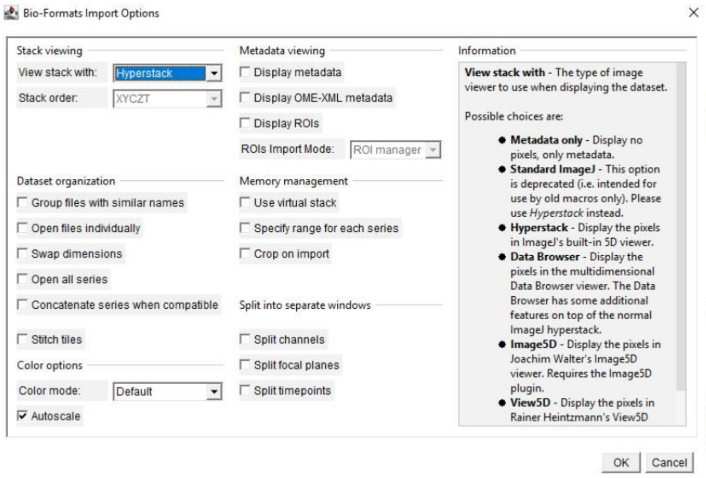


The most frequent types of images ImageJ deals with are

- 8–bit Images that can display 256 (2^8) gray levels (integers only), range of 0–255 (black, 254 grey shades, white).
- 16–bit Images that can display 65536 (2^{16}) gray levels (integers only).
- 32–bit Images that can display 4294967296 (2^{32}) gray levels (real numbers). In 32–bit images, pixels are described by floating point values and can have any intensity value including NaN (Not a Number).
- RGB Color Images that can display 256 values in the Red, Green and Blue channel. These are 24–bit ($2^{3\times8}$) images. RGB color images can also be 32–bit color images (24–bit color images with additional eight bits coding alpha blending values, i.e., transparency).

Opening images (Bio-Formats)

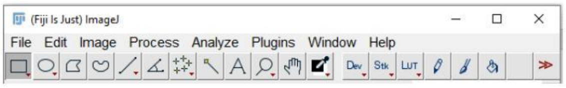
1. Drag an image to the FIJI tool bar or select *File>Open* from the top menu
2. If the image you are opening is a standard format e.g. TIFF, JPG, PNG it should open immediately. If the file type is more obscure you will see the Bio-Formats Import Options window which allows you to set how the image should be opened:
Stack Viewing Determines the window type used to display the image, choose Hyperstack.
Metadata Viewing Opens the standard metadata and/or OME-XML metadata, the latter can be useful when opening OME-Tiffs i.e. Micro-Manager files and will give details of how the image was captured.
Memory Management
1. For datasets too large to be loaded into memory, Virtual Stack is a good option as it only reads the image plane you are viewing into memory at a time.



2. *Specify Range for Each Series* opens only the specified range of image planes as selected by the user. A second dialog will open once the dimensions of the dataset have been analyzed by Bio-Formats. This allows you to select how much of the data you want to open.
3. Crop on import allows the XY dimensions of the image to be cropped to save on memory. In the dialog in the first two boxes you should input the top left corner pixel number of the rectangle you want to crop. The second two boxes tell's bioformats how big the cropped region should be. ***Color Options***
1. Color mode dictates how the channel colors are handled in the images. The most common option is *Composite*. The channels will be merged and open as a single plane, colored according to the image metadata.
2. Colorized will show a plane for each channel in a single window with a scroll bar at the bottom, so you will only be able to see one channel at a time.
3. If *Autoscale* is selected the image intensity display range will map to the min and max data values in the image rather than the bit depth limits of the file format (0-255, 0-4095 etc). Useful for 12- and 16-bit images as often the data is low intensity and is often only several thousand counts, mapping to the full range would make the image hard to see.
Common rules
1. Use tiff files or specific image files, use with cautions png files, do not use compressed jpeg.
2. ImageJ can help you to analyze large complex data, but it won't find more than you. Improve sample quality before image acquisition!
3. 8-bit images are displayed using a grayscale or color lookup table (LUT) => *Image > Color > Display LUT*)
LUT describes the color to be used for each of 256 possible displayed pixel values (pseudo-color)
4. Button «*Undo*» is not like in Office => Use *ctrl+shift+D* to Duplicate image!
Use *Revert* function to return to original image.

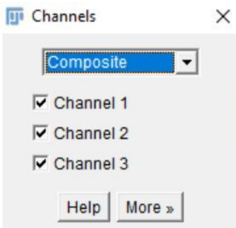
Adjusting the image window size and magnification

1. The size of the image window on the screen will depend on the physical pixel dimensions of the image itself and FIJI will try to optimize it.
2. You can increase/decrease the zoom and size of the image by selecting the *Magnifying Glass* icon. Left click to magnify, Ctrl-left click to de-magnify (% size shown next to the image title).



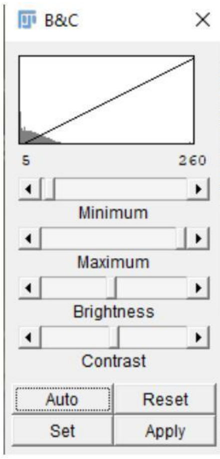
3. When you magnify you'll see two purple rectangles in the top left corner of the image. The outer rectangle represents the whole image and the inner rectangle is the part of the image you can see on the screen. If you want to see more of the image you can go to the bottom right corner of the image and drag it out. Alternatively, if you don't want to increase the amount of screen the image is occupying you can select the hand tool then left click and drag to make another part of the image visible.

Selecting a channel and changing colors

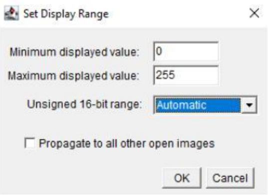


1. Most images will open with the channels merged and in Composite Image mode. Typically, there will be a horizontal scroll bar along the bottom of the image labelled "C" for Channel. Notice when you move this the image doesn't change apart from the color of the text at the top and the bounding box around the image. This is selecting a particular channel of the image for further manipulation e.g. to adjust the brightness of the selected channel only.
2. To physically toggle a channel off go to *Image>Color>Channels Tool*. Then uncheck the channels as required.
3. If you want to change the color of a particular channel, select the channel with the "C" scroll bar along the bottom then select *Image>Lookup Tables* and choose from the list.

Brightness and Contrast



1. Select the channel first as described above then select *Image>Adjust>Brightness/Contrast*.
2. Where you see the histogram the two values on the x axis are the minimum and maximum displayed values. In simple terms moving the maximum scroll bar to the left will make the image brighter and to the left darker. Moving the minimum to the right will make the lower intensity pixels (background) appear to have a higher value or lower if moved to the left.
3. For an 8-bit image pixel intensity values are in the range 0-255 (0=black, 255=white). When you adjust the min and max you should consider these new values as if they were 0 and 255. For example, if the max is set to 208 any pixel with a value =>208 will appear to have an intensity equal to 255 on the display. Likewise, for the minimum, if it was set to 10 then any value <=10 will appear to have an intensity equal to 0.
4. It's important to note that this just changes the image display but does not change the underlying pixel values when you view a histogram or make intensity measurements. However, if you press the *Apply* button this will then set the underlying pixel values to the current display range and will affect intensity measurements but it will only do this for 8-bit images.
5. *Auto* automatically optimizes brightness and contrast based on the image's histogram. It makes a proportion of the pixels saturated. FIJI by default will run the Auto brightness/contrast function when you open an image as well.
6. Press *Reset* to set the display range e.g 0=208 to the full pixel value range e.g. for an 8-bit image 0-255.

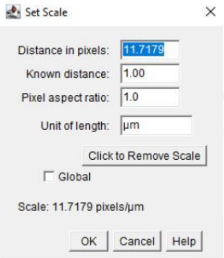


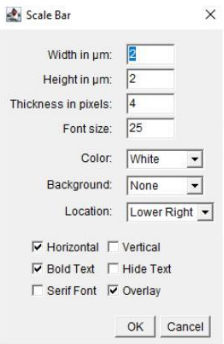
7. How you set the brightness and contrast is important when you want to visually compare the intensity of several images captured with the same exposure time. You should either set them all to the full pixel value range by pressing *Reset* or use the *Set* button to manually set the display range. You can then propagate these values to the other open images.
8. The Unsigned 16-bit range drop down menu allows you to scale the range of grey levels for 16-bit images. E.g. if your 16-bit image has a maximum of 3000 and the display range is set to the pixel value range 0-65535 the image will be very dark, but would be easier to see if you set it to the 12-bit 0-4095 range for example.

Multidimensional images

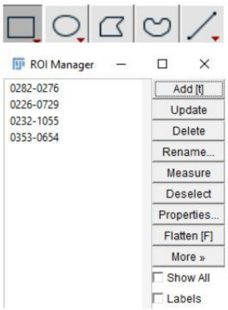
1. When you open an image that includes multiple dimensions e.g. Z, Multi-point and Time you can only look at one image frame at once. There should be horizontal scroll bars at the bottom of the image to allow you to move through each dimension e.g. C, Z, T.
2. Many of the function's relating to image stacks are found in *Image>Stacks.*

Image properties, setting scale and scale bars





1. Select *Image>Show info* and you will see the metadata of the image. If you need to find out a particular detail about the image or how it was taken this is where to do it.
2. Images with no scale set will only list dimensions in pixels at the top of the image. If a scale is set you will also see the dimensions in µm.
3. If you need to set or adjust the scale go to *Analyze>Set Scale*.
4. If a scale is set you can see it at the bottom of the window. To adjust the scale set the Distance in Pixels to 1. The known Distance is the number of µm 1 pixel represents. You need to know this figure in order to set the scale correctly.
5. Once the scale is set you can add a scale bar to the image through *Analyze>Tools>Scale Bar*.
6. You can change various aspects of the scale bars appearance most of which is self-explanatory. If Overlay is selected then the scale bar is added as an overlay. This is a non-permanent addition to the image which doesn't affect underlying pixel values. You can hide or show an overlay at any time using *Image>Overlay>Show/Hide Overlay*. It will only be accessible when you open the image in FIJI. If Overlay is not ticked then the scale bar is "stamped" onto the image and is permanent and cannot be removed. It also becomes part of the pixel data and will affect any intensity-based measurements.



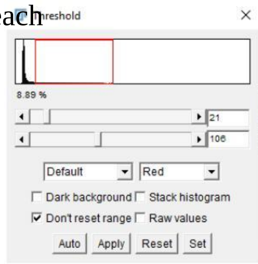
Selections and ROIs (ROI Manager)

1. A Selection is essentially an ROI. They are used to isolate a part of the image e.g. if you want to crop the image or if you want to measure only within a defined area. You can use any of the selection tools to draw a selection on the image.
2. If you want to add multiple selections to the image and recall them at any point, use the ROI Manager (*Analyze>Tools>ROI Manager*).

3. Draw a selection then press Add to store it, a row will be added to the ROI Manager table. You can add multiple selections to the image in this way. Clicking on a row will make it active and show it on the image.
4. Change the color of selections using *Edit>Options>Colors*

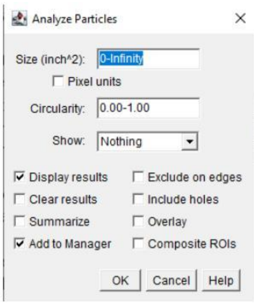
Image Wide and Selection Based Measurements

1. Select *Analyze>Set Measurements*, here you can decide what measurements you want to make.
2. Limit to threshold applies if a threshold has been applied to the image, see sections below.
3. Add to overlay creates a removable layer on the image which will highlight what has been measured, this can be toggled off/on via *Image>Overlay>Show/Hide Overlay*.
4. Redirect To enables selection of any open image and the measurements will be done on the image selected 5. To calculate these measurements, select *Analyze>Measure*.
6. If you draw a selection before pressing measure only the pixels within the selection will be measured. 7. A table of the results will open, save it using *File>Save As*.
8. To summarize the results and get the mean, SD, minimum and maximum of the values in each column select *Results>Summarize*.



Identification of Objects and Measurements (Thresholding)

1. Setting a threshold is a means to segment images into features of interest and background. It is intensity based and involves isolating intensities of interest using the image histogram.
2. Select *Image>Adjust>Threshold*. An automatic threshold will be calculated and any pixels included in the threshold will be colored red. Those pixels fall within the red box now on the histogram in the threshold window.
3. You can expand/reduce the limits of the threshold to include more or less intense pixels by moving the horizontal scroll bars. Press *Auto* to go back to the auto calculated threshold.
4. *Reset* will remove the threshold altogether.
5. *Apply* will binarize the image i.e. all pixels in the threshold become black, a value of 0 and all pixels in the background become white, a value of 255. Using *Apply* isn't necessary, just close the threshold window and the threshold will still be visible on the image.
6. Set the measurements you want to calculate as described earlier then select *Analyze>Analyze Particles*.
7. The size box is a size filter, by default any object size is accepted so long as it falls within the threshold. Note the size is the area in pixels.
8. Circularity filters how close to a perfect circle the objects are, where 1 is a perfect circle. This is difficult to set without having a preview of how this affects the objects included in the threshold.
9. The *Show* drop down determines how the identified objects will be displayed, *Outlines* is useful as it creates a new image with no data other than the outline of each object a number to identify it.
10. Display *Results* will show the results table and associated values from all objects.



11. *Exclude on edges* and *Include holes* will exclude any objects touching the image boundary and will include any objects where the threshold encompasses the object but an area internally is not within the threshold respectively.
12. Selecting *Add to Manager* will add all the identified objects as separate rows in the *ROI Manager*. This allows selection of any of the objects for further individual or batch processing.
13. You could delete all objects not meeting your criteria by holding ctrl click to select them then press *Delete*. Then measure the remaining again by selecting *Show All* and press *Measure* from the *ROI Manager*.

Examples

A. Unmixing of objects with specific spectra

Nuance file **.im3** can't be opened in ImageJ using **BioFormat** plugin if reference cube was used. Therefore, cubes should be saved as a tiff cubes in Nuance.

1. *File > Import > Image Sequence > Select **your folder***.
2. *Image > Type* and select 8-bit to convert all slices in the stack (wavelengths are indicated)
3. *Plugin > Install > Depth Color Code 0.0.2*, MSA_514, and Compute. Select Plugins folder in Fiji.app
4. *Plugin > Stacks > Z stack > Depth Color Code 0.0.2*. Use Rainbow RGB
5. *Image > Stacks > Z project* (rendering)
6. *Image > Stacks > Plot Z-axis profile* (point, line, box) to see spectra of the objects, use ROI manager
7. *Plugin > MSA 514*. Number of factorial images 4 (For uniform background reference values of illumination should be acquired)
8. Multivariate Statistical Analysis of image series https://imagej.nih.gov/ij/plugins/inserm514/
   a) Principal Component Analysis (PCA) b) Correspondence Analysis (CA)
9. *Ctrl+Shift+D* to duplicate target component images and then make Composite image
10. *Image > Adjust > Threshold*; *Image > LUT > Red/Blue*; *Image > Color > Merge channels*

B. Quantification of area

1. *File > Open **your file***. *Ctrl+Shift+D* to duplicate image.
2. *Image > Type* and select 8-bit. Color values are averaged or *Image > Color > Split Channels*. Select the corresponding channel (8-bit).
3. Straight selection. *Analyze > Set scale (mm)*. Can be saved
4. *Image > Adjust > Threshold.* Use the same values in all images
5. Wand tool (Tolerance). *Analyze > Set Measurements*. Measure (see Help)
6. Copy results
7. *Analyze > Analyze Particles*. Select the 'Add to Manager'
8. **Create macros for batch analysis (Plugins > Macros > Record)**
   Repeat all the steps (*Image Type*, *Threshold*, *Analyze Particles)*. Do not close Threshold window. After press *Create*
9. **Use new macros in batch analysis (Process > Batch).** Choose the folder with your images, you want to analyze and your created macros.

C. Quantification of Live/Dead Staining

1. Split channels: live cells are in the Green Channel and dead cells are Red Channel. Discard the Blue Channel. Channels must be analyzed separately for live and dead fluorescent channels (*Image > Color > Split Channels*)
2. Work with one channel at a time. Convert the channel to 8-bit (if they are not yet in that color graphic format). This is done so that the images may be threshold based on intensity (*Image > Type > 8-bit*)
3. Select the *Find Maxima* function from each channel to count number of dead or live cells. Select the *Point Selection* output type and check the box for *Preview Point Selection*. Adjust the *Noise Tolerance* values by increments of 5 or 10 until background staining is excluded (Be mindful of adjusting the tolerance value so the red and the green "points" do not overlap. However, some overlap may occur and it is dependent on the user to determine how much overlap is acceptable). The number of points will be the total number of cells positive for the stain of interest (numbers are based on user's definition of Live/Dead cells) (*Process > Find Maxima*)

Use the following formulas for quantification:

Total Cell Number = Live Cells + Dead Cells

Percentage of Live Cells = (Live Cells/Total Cell Number) *100

Percentage of Dead Cells = (Dead Cells/Total Cell Number) *100

https://imagej.net/software/fiji/

Fiji is an image processing package—a "batteries-included" distribution of ImageJ2, bundling a lot of plugins which facilitate scientific image analysis

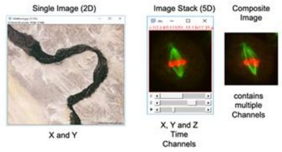
Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., … Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. Nature Methods, 9(7), 676–682. doi:10.1038/nmeth.2019
+ plugin references

- ImageJ and its Java source code are freely available and in the public domain
- Automate tasks and create custom tools using macros
- More than 500 plugins are available
- Edit, and measure of point, line and area selections
- Image enhancement and geometric operations
- Measure area and density
- Color processing
- Process a "stack" of spatially or temporally related images (slices) in a single window

Photoshop: common photo

Power Point: flowchart

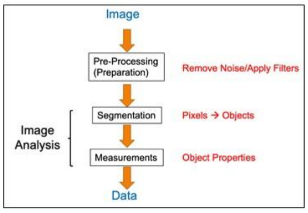
ImageJ: microscopy



Common rules

- **Use .tiff files**, **use with cautions .png files**, **do not use compressed .jpeg**
- Corrections => Measure. Objective fast quantitative result
- ImageJ can help you to analyze large complex data, but it won't find more than you

  Improve sample quality before image acquisition!



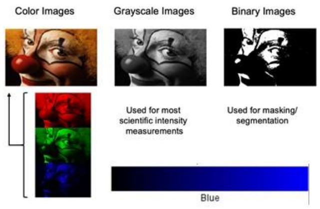
- 8-bit image = > pixel intensity is in the range of 0–255 (black, 254 grey shades, white)
- Grayscale images are displayed using a color lookup table (LUT)

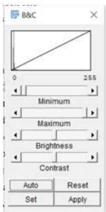
  LUT describes the color to be used for each of 256 possible displayed

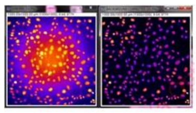
  pixel values (pseudo-color)
- Button «**Undo**» is not like in Office =>

  Use **ctrl+shift+D** to Duplicate image!

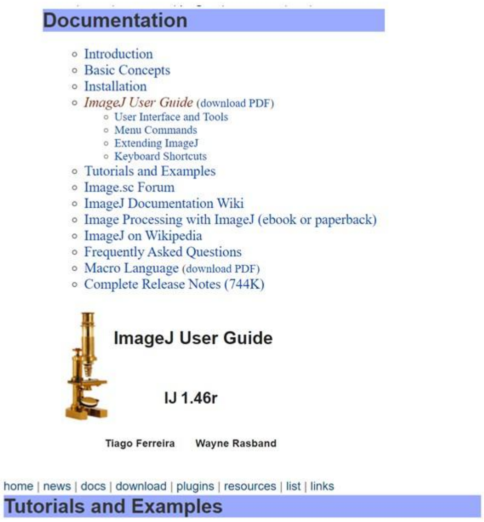
  Use Revert function to return to original image



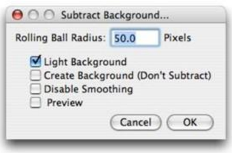




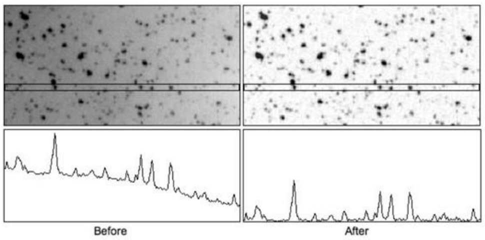
Help



Subtract Background



The *Rolling Ball Radius* is the radius of curvature of the paraboloid. As a rule of thumb, for 8-bit or RGB images it should be at least as large as the radius of the largest object in the image that is not part of the background. Larger values will also work unless the background of the image is too uneven. For 16-bit and 32-bit images with pixel value ranges different from 0-255, the radius should be inversely proportional to the pixel value range. For example, typical values of the radius are around 0.2 to 5 for 16-bit images (pixel values 0-65535).

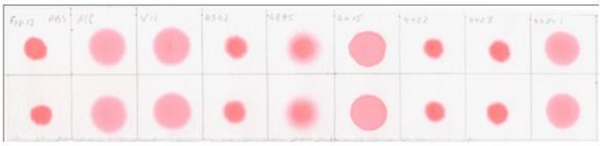


Particles (cell, aggregates) analysis

1. Spot quantification: Area

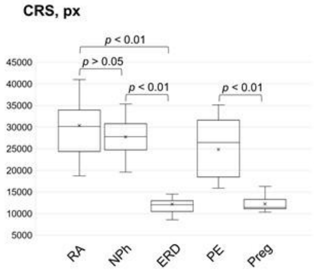
"The greatest scientists are also artists"
Albert Einstein

CRD paper test to assess proteinuria
Congo red dye is added to urine and mix is applied on paper
The more proteinuria, the more area of spots



- File > Open and select your file (RGB.tif). C**trl+Shift+D**
- Image > Type and select 8-bit. Color values are averaged
- Straight selection. Analyze > Set scale (mm). Can be saved
- Image > Adjust > Threshold. Use the same values in all images
- Wand tool (Tolerance). Analyze > Set Measurements. Measure (see Help)
- Copy results
- *Analyze > Analyze Particles. Select the 'Add to Manager'
- ***Create macros for batch analysis (Macros > Record)***

  do not close Threshold window. Create
- ***Use new macros in batch analysis (Process > Batch)***



home | news | docs | download | plugins | resources | list | links

ImageJ Macro Language

Particles (cell, aggregates) analysis

2. Live/dead quantification: Split and Count

Live cells are stained with calcein and generate **green fluorescence** upon the excitation of their cytoplasm.
Dead cells are labeled with the ethidium homodimer dye (EthD) which binds to their DNA and **fluoresces red.**

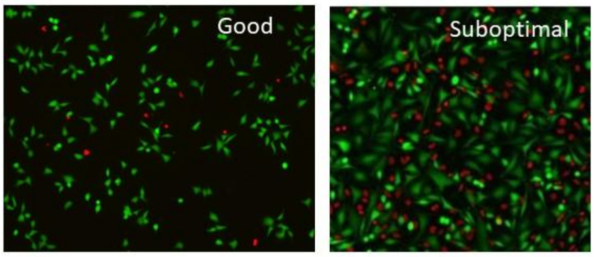
- File > Open and select your file (RGB.tif). See Image > Properties
- Analyze > Tool > Scale bar
- Image > Overlay > Flatten
- Image > Color > Split Channels. Select the green channel (8-bit)
- Image > Adjust > Threshold. Apply to get binary image (cell fragment)
- Process > Binary > Watershed
- Analyze > Analyze Particles

  Select Summarize the 'Add to Manager' for corrections
- Repeat the procedure for the red channel
- Calculate the percentage of live cells

  ! Compare with multipoint tool and Cell Counter (Plugin > Analysis)

  ! Compare with live+dead+quantification macros

Live= **209** ; Dead= **11** ; Viability= **5 %**



Microscope images of Live/Dead assay of neural stem cells

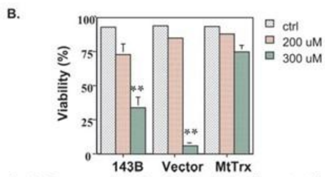


FIG. 6. **Overexpression of mtTrx in 143B cells protected against tBH-induced cytotoxicity.** *A*, flow cytometry analysis of cell

Chen et al., 2002

3. Aggregate quantification: Count and Area

Particles (cell, aggregates) analysis

Amyloidogenic **PrP** fused to GFP forms dot **aggregates** with green fluorescence in cytoplasm of yeast cells with cell walls
GFP alone does not aggregate and gives rise only **diffuse** green fluorescence

Bright field

- File > Open and select your file (RGB.tif)
- Image > Type and select 8-bit
- Process > Find Edges or Subtract background (10)
- Image > Adjust > Threshold
- Process > Binary > Close, Fill holes, Watershed
  (note Pan Edges when erode)
  Select and Measure smallest cell
- Analyze > Analyze Particles. Select Summarize
  ! Compare with Auto local threshold
  The segmented phase is always shown as white (255).

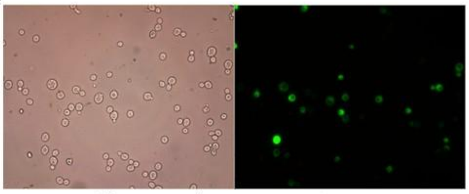
Fluorescence

Aggregates

- File > Open and select your file (RGB.tif)
- Image-Color-Channels Tool
- Process > Image calculator*. Overexposure in RGB
- Process > Find Edges
- Process > Binary > Make binary
- Analyze > Analyze Particles. Select Summarize

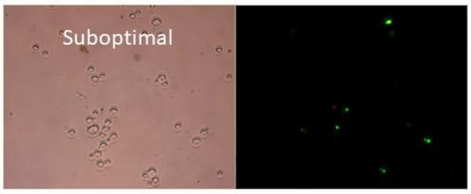
Cells

- Image > Adjust > Brightness/Contrast
  !!! Be consistent with your adjustments
  Non-linear adjustment in Process > Math > Gamma
- Process > Filters > Unsharp mask 
  ! can affect the color in the image
  Compare with Bandpass filter and Log3D (install)
- Image > Color > Split, Adjust > Threshold
- Process > Binary > Close, Fill holes, Watershed.
- Select and Measure smallest cell
- Analyze > Analyze Particles. See Redirect + Overlay



Diffuse GFP fluorescence in yeast

Count:	All Cells = 92	Green cells = 46	Green dot = 0
Area:	All Cells = 30539	Green cells =10302	Green dot =0



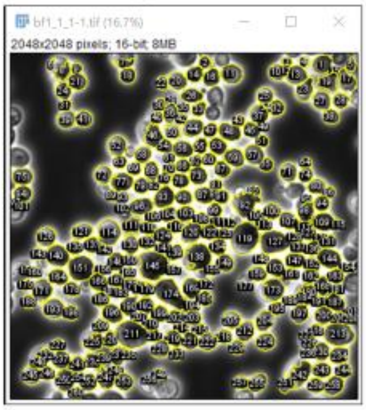
Dot of PrP-GFP aggregates in yeast

All Cells = 44	Green cells = 12	Green dot =21
Cell area=20156	Green cells =3328	Green dot = 631

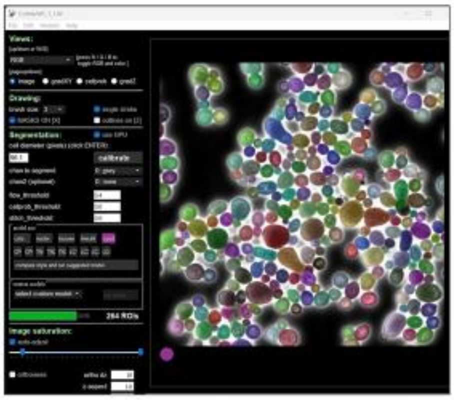
Trainable Weka Segmentation plugin

https://forum.image.sc/latest
Scientific Community Image Forum is a discussion forum for scientific image software

Cellpose https://www.cellpose.org/
a generalist algorithm for cellular segmentation written in Python 3



VS



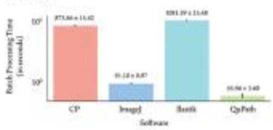
Investigation of Different Free Image Analysis Software for High-Throughput Droplet Detection

Table 2. CP Gives the Highest Accuracy and Precision

Category	CellProfiler	ImageJ	Ilastik	QuPath
% Accuracy	96.2%	92.7%	74.7%	80.9%
% Precision	99.8%	96.3%	80.2%	83.1%

Sanka et al., 2021 https://pubs.acs.org/doi/10.1021/acsomega.1c02664

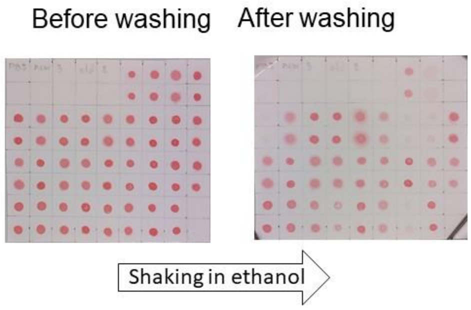
Figure 4

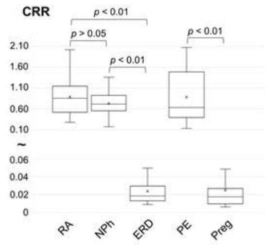


1. Spot quantification: density/intensity

Density calculations

CRD membrane test to assess proteinuria
Congo red dye is added to urine, mix is applied on membrane which is washed in ethanol
The more proteinuria, the more brightness of spots





- File > Open and select your file

  RGB camera is used because CRD is semiqualitative
- Image > Stacks > Tool > Combine
- Image > Type and select 8-bit

  converting RGB image to grayscale (Edit > Options > Conversion)

unweighted: gray level = (R + G + B)/3	weighted: gray level = 0.3R + 0.59G + 0.11B

- Process > Subtract background
- Rectangle, push 1, (duplicate, push 2)x3, push 3 (Analysis > Gel)
- Straight, separate peaks, Wand tool, measure areas
- Copy results

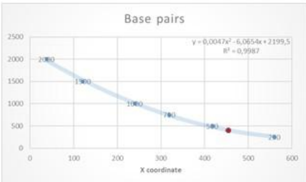
2. Concentration and size quantification: Intensity and calibration

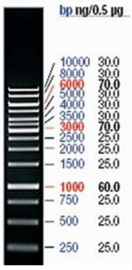
Density calculations

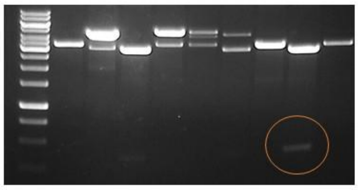
DNA, RNA, protein fragments are separated in the gel and stained with ethidium bromide

Concentration quantification is more specific in comparison with spectrophotometric assays. For some protocols density quantification is the only possible methods for concentration measurements (sample in detergents)

Pixels must be unsaturated!







Agarose electrophoresis of DNA fragments
At the left DNA ladder is loaded
Thermo Scientific™ O'Gene Ruler

Size

- File > Open and select your file
- Image > Type and select 8-bit
- Process > Subtract background
- Rectangle, push 1, duplicate, push 2, push 3 (Analysis > Gel)
- Multipoint tool, measure peaks, save X coordinates
- Calculate band size in Excel (414 base pair)

Concentration

- File > Open and select your file
- Image > Lookup Tables > HiLo

  Shows saturated pixels in red and minimal value pixels in blue
- Repeat steps 2-4 in Size measurements
- Straight, separate peaks, Wand tool, measure areas
- Calculate band mass and molar proportion in Excel

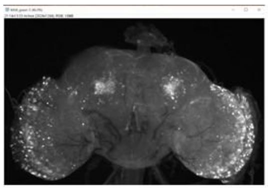
  (7.67 ng/µl, 1500/413=3.6, 36ng/25 = 10ng/7.67)

3D images: restacking and annotations

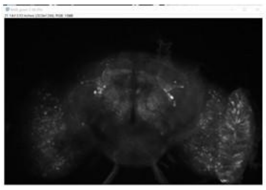
Drosophila brain slices with GFP fluorescence in neurons
Whole-mount immunohistochemistry
Confocal laser microscopy

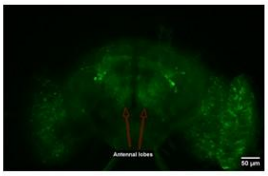
- File > Open Zeiss file (.CZI). Plugin Bio-Formats Importer
- Image > Hyperstacks > Make subset (define channel and slices)
- File > Save as > Image Sequence
- File > Import > Image Sequence
  ! Compare Image properties
- Image > Stacks > Z project (define slices)
- Image > Type and select 8-bit
- Image > Lookup Tables > Green
- Arrow, Color Picker. Image > Overlay > Add selection
  Compare Overlay options and Arrow tool options
  See Edit > Draw (Arrow, Color Picker)
- Add arrow in ROI manager to copy. Move arrow, Add selection, Add ROI
- Repeat with Text and Scale bar and Image > Overlay > Flatten
- Save as tiff filed (Set image resolution and sizes)
- See Plugins > 3D Viewer or Image > Stacks > 3D projects (Speed option)

1-26 slices



19-26 slices

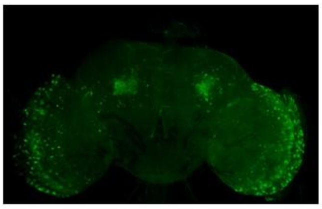




Stacks and slices

Operations in stack

- File > Open Zeiss file (.CZI)
- Crop acts to all slices
- Cut, Math, Image calculator, Filters can act to each slice
- 3D Filter acts to all slides
- Overlay options can be change to acts to all slices
- Image > Stacks > Measure stack
- Image > Stacks > Tool > Plot XY Profile
- Image > Stacks > Plot Z-axis Profile
- Image > Stacks > Orthogonal views



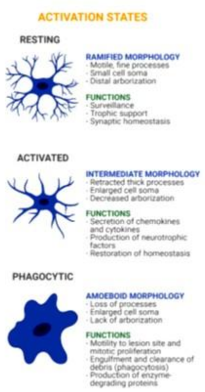
Morphology quantification

jove Journal of Visualized Experiments

Video Article

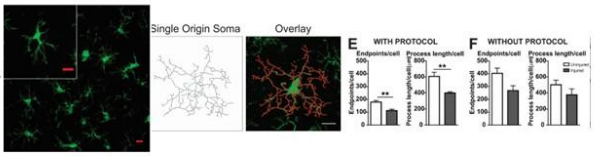
Quantifying Microglia Morphology from Photomicrographs of Immunohistochemistry Prepared Tissue Using ImageJ

Kimberly Young[1], Helena Morrison[1]
[1]College of Nursing, University of Arizona



Microglia are brain phagocytes that undergo a broad range of morphologic changes. ***AnalyzeSkeleton*** and ***FracLac*** summarize cell morphology in terms of process endpoints, junctions, and length as well as complexity, cell shape, and size descriptors. The protocol can be used to stratify between diverse microglia morphologies present in the healthy and injured brain.

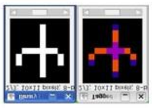
Microglia stained by Ca2+ ATPase method, Lab of Armen Voskanyan



- File > Open and select your file (RGB.tif). Stack is also applicable
- Process > FFT > Bandpass filter (to remove small features)
- Image > Type and select 8-bit

? Brightness and contrast are changed by updating the image's lookup table (LUT), so pixel values are unchanged.

- Process > Filters > Unsharp mask (radius 3, weight of 0.6)
- Process > Noise > Despeckle (median filter 3x3)
- Image > Adjust > Threshold
- Process > Noise > Despeckle
- Process > Binary > Close (1,1)
- *Process > Noise > Remove Outliers (bright)*



End-point voxels: less than 2 neighbors.
Junction voxels: more than 2 neighbors.
Slab voxels: exactly 2 neighbors.

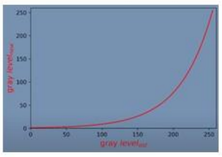
! bright outliers are targeted with a pixel radius of 2 and a threshold of 50

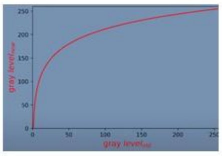
- Duplicate image. Process > Binary > Skeletonize
- Analyze > Skeleton > Analyze Skeleton (2D/3D)

Assess for accuracy by creating an overlay of the skeleton and the original image
Circular somas should be avoided through protocol adjustment
Use Line tool to determine length of fragments to be trimmed from the dataset
! Try protocol with Image from Voskanyan's lab adding Fill Holes
Try FracLac with binary cells. Use Process > Binary > Outline. See circularity

ImageJ Editing

- Selection options
- Threshold (over/under)
- Select region. Analyze > Analyze Particles
- Set measurements. Area fraction
- Options in Analyze > Histogram and Plot Profile
  Comparisons of expression level. Specify with Threshold
- Analyze > Calibrate (function choice)
- Analyze > Tools > Calibration bar
- Image > Transform > Image to results
  If you use Image calculator with 8-bit images than you should escape results outside min and max
- Process > Math (Macro option)
- Process > Filters > Convolve (Smooth. Mean, Gaussian Blur)
  Show circular masks





Plugins

https://forum.image.sc/latest
Scientific Community Image Forum is a discussion forum for scientific image software sponsored by the Center for Open Bioimage Analysis (COBA).

HyperSpectral plugin

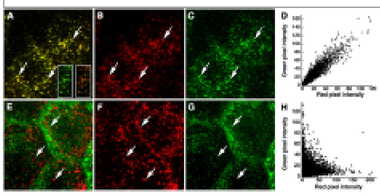
Trainable Weka Segmentation

TrackMate

In perspective

- Colocalization
- Transmission electron microscopy

Colocalization of fluorescent markers: folder "Coloc_3D"



Transferrin conjugated to Texas Red (B) and Alexa 488 (C). Arrows indicate endosomes containing both probes

Texas Red-dextran (F) and Alexa 488-transferrin (G). Arrows indicate examples of lysosomes containing Texas Red-dextran

!!! Colocalization

- cannot indicate that two proteins/molecules are bound or interacting, only that they are both localized to within a certain volume, and is mostly dependent upon your **microscope and its acquisition parameters**
- is most often used to determine if a protein is localizing to an organelle or other well defined cellular structure

Plugins: Coloc2, JaCoP, Colocalization finder

Pixel-wise methods

- Pearson's correlation coefficient
- Manders split coefficients (Costes threshold)

colocalization analysis

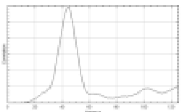
JaCoP, Distance Analysis

Object-based colocalization

Colocalization by cross-correlation, JaCoP, ImagingFCS

Cross-correlation function

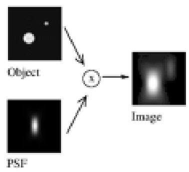
- Spatial cross-correlation
- Temporal cross-correlation



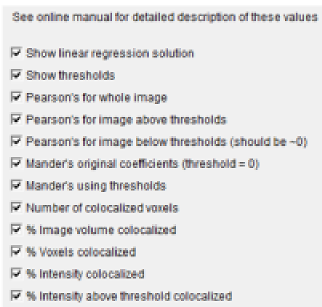
\+

significance tests based on randomization

Point Spread Function (PSF) describes what a single point in the object looks like in the image



- File > Open > colocsample1bRGB_BG
- Define ROI: Image > Type > 8-bit, Image > Stack > Z Project, Image > Adjust > Threshold, Process > Binary > Fill holes
- Select with Wand tool and add to ROI manager (t)
- Image > Color > Split Channels. Select the green and yellow channels (8-bit). Process > Subtract background
- Analyze > Colocalization > Colocalization Threshold/Test/Coloc 2



https://imagej.net/imaging/colocalization-analysis

Unmixing of objects with specific spectra: folder "HSI"

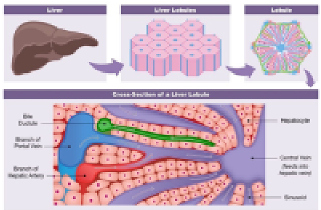
Liver lobules in a slice have a clearly visible central vein and barely visible branches of the veins flowing into the central vein.
HS analysis helps to segregate small vein branches

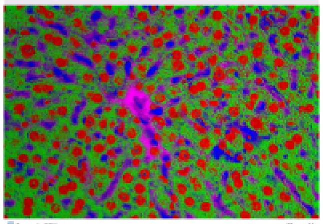
Nuance file **.im3** can be opened in ImageJ using **BioFormat** plugin (takes time)
The 16-bit slices were converted to 8-bit and saved as Image Sequence (stack)
The Wavelength range and step were indicated in the name of the stack

- Plugin > Install > ZstackDepthColorCode_-0.0.2 and MSA_514. Select Plugins folder in Fiji.app
- File > Import > Image Sequence
- Image > Stacks > Z project (point, line, box)
- Plugin > Stacks > Z stack > Depth Color Code 0.0.2. Use Rainbow RGB
- Image > Stacks > Z project (rendering)
- Plugin > MSA 514. Number of factorial images 4

  Multivariate Statistical Analysis of image series https://imagej.nih.gov/ij/plugins/inserm514/

  a) Principal Component Analysis (PCA) b) Correspondence Analysis (CA)
- **ctrl+shift+D** to duplicate target component images and then make Composite image
- Image > Adjust > Threshold; Image > LUT > Red/Blue; Image > Color > Merge channels





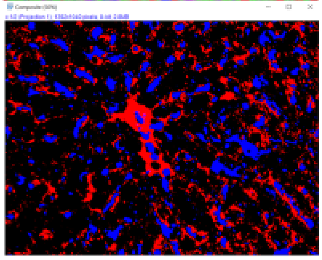


IMAGE PROCESSING QUIZ

1.What does the plugin "Analyze Particles" in ImageJ/FIJI do?

A. It analyzes the color composition of the image
B. It measures and identifies particles in binary or thresholded images
C. It smooths the image by removing noise
D. It rotates the image

2. What is the purpose of the "Split Channels" option in ImageJ when working with color images?

A. It separates the image into its constituent color channels.
B. It creates multiple copies of the image.
C. It enables the user to apply different filters to each channel.
D. It reduces the size of the image file

3. What does the "Threshold" function in ImageJ/FIJI do?

A. It differentiates the background from the objects of interest based on intensity
B. It calculates the mean intensity of the image
C. It merges multiple images
D. It saves the image to a specified location

4. Which language is the ImageJ software written in?

A. C++
B. Python
C. Java
D. Lisp

5. How can you automate a repetitive task in ImageJ/FIJI?

A. By repeatedly clicking the "Play" button
B. By using the "Force Automation" command
C. By recording a macro and replaying it when required
D. By downloading additional automation software

6. What feature in ImageJ/FIJI can assist in improving the quality of acquired images?

A. The "3D Viewer" function
B. The "Calculator Plus" plugin
C. The "Despeckle", "Smooth" or "Denoise" functions
D. The "Time Stamper" plugin

7. How can batch processing be carried out in ImageJ/FIJI?

A. By opening multiple images at once.
B. By using macros and applying them on a series of images using “Process > Batch > Macro”.
C. By connecting multiple devices and performing simultaneous processing.
D. Batch processing is not possible in ImageJ/FIJI.

8. In the context of ImageJ/FIJI, what does ROI stand for?

A. Return On Investment
B. Region Of Interest
C. Range Of Intensity
D. Radius Of Inertia

9. What distinguishes ImageJ/FIJI in terms of cost from many other image processing and analysis software?

A. It offers a 30-day free trial.
B. It provides discounts for students and educators.
C. It uses a subscription-based pricing model.
D. It is an open-source software, available for free.

10. Which of the following operations can be performed using the ImageJ software?

A. Processing images in multiple formats
B. Macro scripting to enhance its functionality
C. Image segmentation
D. Creation of density histograms and line profile plots
E. All of the above

ANSWERS TO QUIZES

CELL STAINING

1. A
2. C
3. B
4. A
5. D
6. C
7. B
8. A
9. E
10. B

CONFOCAL MICROSCOPY

1.A
2.B
3.C
4.B
5.C
6.D
7.B
8.C
9.B
10.B

ATOMIC FORCE

1. D
2. B
3. D
4. E
5. D
6. E
7. A
8. E
9. E
10. B

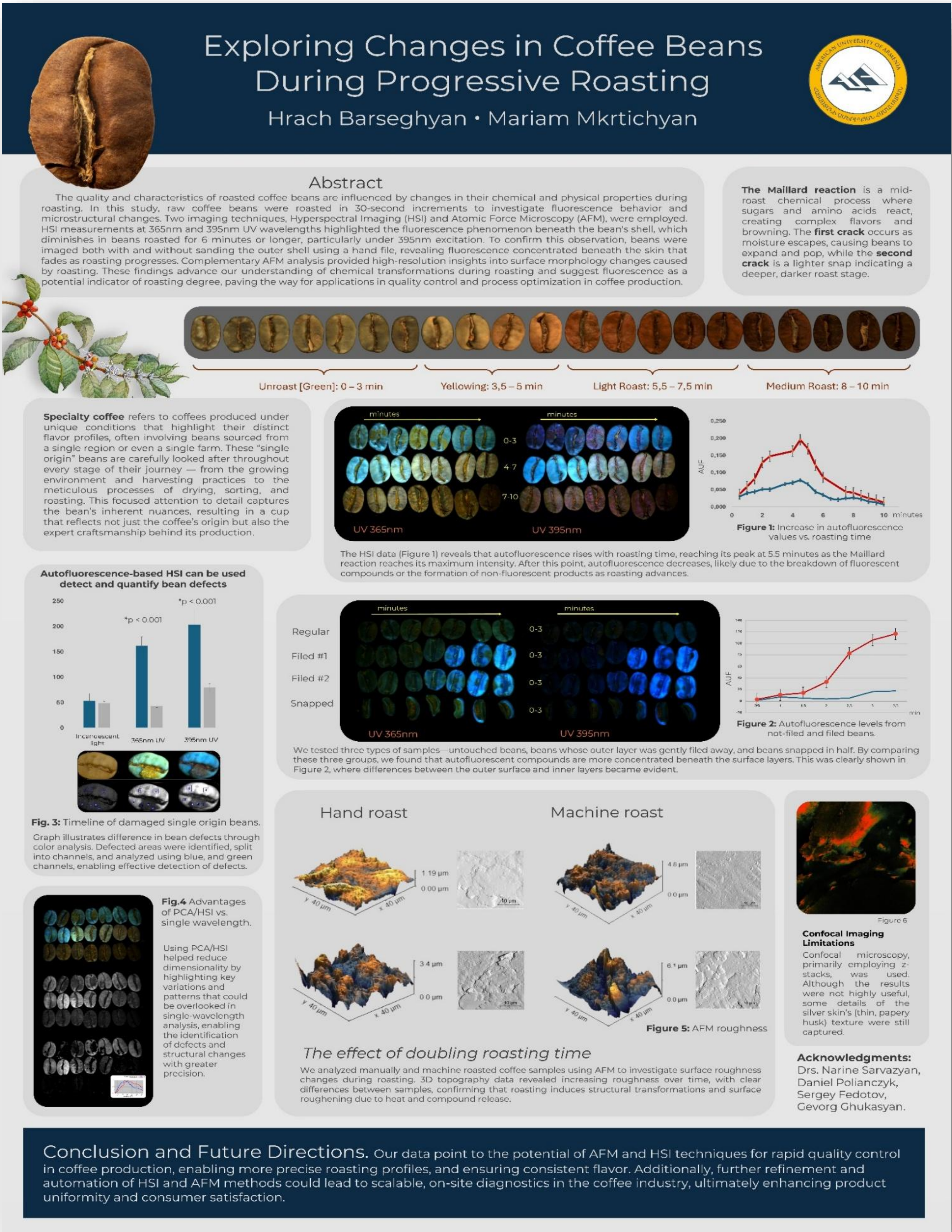
HYPERSPECTRAL IMAGING

1.A
2.C
3.C
4.D
5.C
6.B
7.D
8.D
9.D
10.B

IMAGE PROCESSING

1.B
2.A
3.A
4.C
5.C
6.C
7.B
8.B
9.D
10.E

EXAMPLES OF STUDENT POSTERS



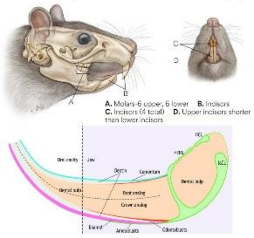
COMPARING TEETH FROM DIFFERENT SPECIES

Anna Ohanyan[1], Fernando Villarruel[2]
[1] American University of Armenia, [2] L. A. Orbeli Institute of Physiology, NAS RA

Rationale: Teeth are critical biological structures that provide insights into health, diet, and evolutionary adaptations across species. Advanced imaging techniques enable detailed analysis of their composition, structure, and functional properties. This study explores the microstructural and compositional characteristics of different teeth: gyurza snake (*Macrovipera lebetina obtusa*) fang, rat (*Rattus norvegicus domestica*) down incisor, and human (*Homo sapiens*) molar. The study included three Imaging Methods: **(1)** Laser Scanning Confocal Microscopy (LSCM), **(2)** Atomic Force Microscopy (AFM), and **(3)** Hyperspectral Imaging (HSI), allowing to reveal structural and biological details, surface property analysis and characterization of the materials in the teeth by analyzing their reflectance/autofluorescence profiles, respectively. By comparing teeth from distinct species, we aim to identify common and unique features that highlight functional adaptations and their significance.

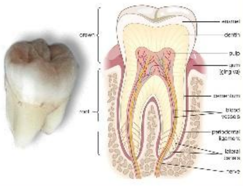
Samples description:

Snake: Most snakes have quite long, thin, sharp, and recurved teeth for catching and eating prey. These teeth are firmly anchored to the anterior bone on the lower jaw and the upper jawbone. Just like sharks and crocodiles, snakes replace their teeth throughout their life. Teeth are shed in stages and the snake will still have enough teeth present to eat, even when some are shed.[1]



Rat: There are two types of rat teeth: incisors and molars. Incisors are the big rat teeth that continuously grow. There are four incisors in total—two on the top and two on the bottom—directly in the front of the mouth. The rest of a rat's teeth are just like human teeth. Molars line the inside of the mouth and do not continuously grow once they are formed.[2]

Human: Human teeth function to mechanically break down items of food by cutting and crushing them in preparation for swallowing and digesting. Humans have four types of teeth: incisors, canines, premolars and molars, which each have a specific function. The incisors cut the food, the canines tear the food and the molars and premolars crush the food. [3]

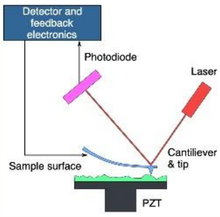


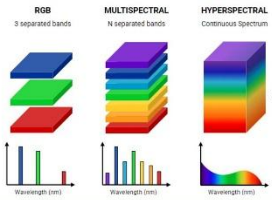
Imaging Techniques description:

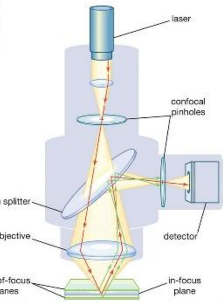
Atomic Force Microscopy: Atomic Force Microscopy is a very high-resolution type of scanning probe microscopy with demonstrated resolution on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit. The information is gathered by "feeling" or "touching" the surface with a mechanical probe. The AFM three major abilities: force measurement, topographic imaging, and manipulation, make it a powerful method of investigation and visualization of a various life science targets.[2]

Hyperspectral imaging: Hyperspectral imaging is a powerful new technology based on spectroscopy. It is based on collecting hundreds of images at different wavelengths from the same spatial area. The collected data form a hyperspectral cube, in which two dimensions represent the spatial coordinates (x,y), while the third coordinate shows wavelength (λ). Hyperspectral imaging yields the spectrum for each pixel in the image, making it possible to find objects, identify materials, or detect hidden text or images otherwise invisible to a naked eye using a variety of mathematical algorithms.[3]

Confocal Microscopy: The primary functions of a confocal microscope are to produce a point source of light and reject out-of-focus light, which provides the ability to image deep into tissues with high resolution, and optical sectioning for 3D reconstructions of imaged samples. The basic principle of confocal microscopy is that the illumination and detection optics are focused on the same diffraction-limited spot, which is moved over the sample to build the complete image on the detector. [1]



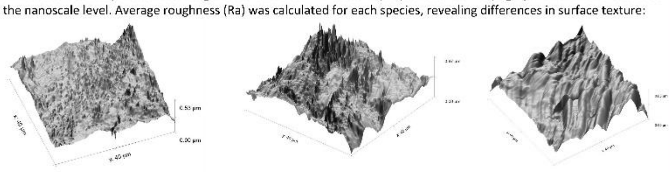




AFM:

Surface roughness analysis:

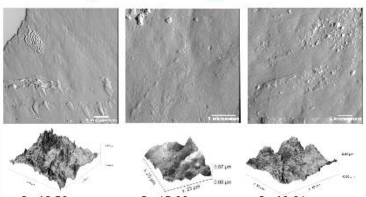
AFM was used to study the surface roughness of the teeth. This technique provided a 3D topographical view of the surface at the nanoscale level. Average roughness (Ra) was calculated for each species, revealing differences in surface texture:



Snake (Ra 13.53 nm) **Rat** (Ra 15.23 nm) **Human** (Ra 28.85 nm)

Snake tooth has the smoothest surface, indicating minimal surface irregularities or wear. Rat tooth has moderate roughness, suggesting more pronounced surface features compared to snake tooth. Human tooth has the roughest surface, which might be linked to its functional demands and enamel structure. The smoother surface of snake teeth could aid in reducing friction when capturing and consuming prey.

Variability between different rat individuals:



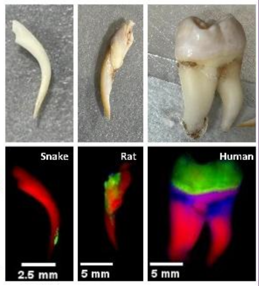
Ra 13.53 nm Ra 15.23 nm Ra 13.81 nm

For rat teeth, measurements from three different samples showed slight variability in average roughness values with Ra mean and standard deviation of 14.19 and 0.74, respectively (individual Ra showed in figure). This variation highlights the natural differences between individual teeth, possibly influenced by factors such as wear, diet, or biological variability. Despite the differences, rat teeth consistently exhibited intermediate roughness compared to the smoother snake teeth (13.53 nm) and the rougher human teeth (28.85 nm).

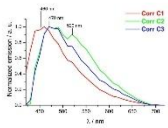
HSI:

Autofluorescence HSI:

HSI was used to analyze teeth fluorescence. Samples were excited with a 395 nm light source and fluorescence emission was scanned in the 420-720 nm range. The human tooth image was unmixed using NUANCE software, obtaining 3 components relative contribution for each pixel of the image. Components spectra were corrected by equipment sensitivity on each wavelength, for comparison with those reported in bibliography.

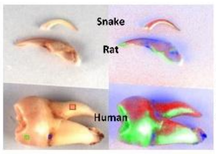


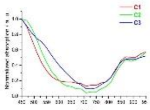
Previously reported fluorescence spectra in the literature (exc at 395 nm) showed a wide maximum at ~ 500 nm. In our study, dentin spectrum was consistent with that but enamel showed maximum displaced to UV region (460 nm).[7]



Brightfield HSI:

Brightfield HSI was used to analyze differences in absorbance between different teeth and/or teeth parts. Results showed that, due to the fact is highly reflective (white), brightfield HSI led to non informative results. Briefly, spectra from components are very similar and their relative contribution in image is related with differences in illumination rather than in tissue characteristics.

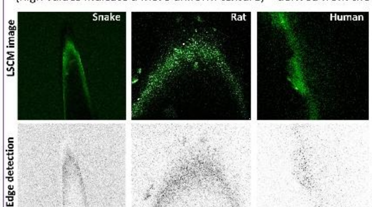


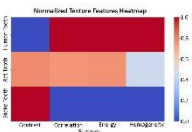


LSCM:

Texture analysis:

Using confocal microscopy, we analyzed the structural and textural properties of human, rat, and snake teeth to understand their microstructural differences. This technique allowed us to quantify key texture features—Contrast (high values indicate large differences between neighboring pixels), Correlation (high values indicate a more predictable structure) , Energy (high values indicate a more uniform texture), and Homogeneity (high values indicate a more uniform texture)—derived from the grayscale images of the teeth.





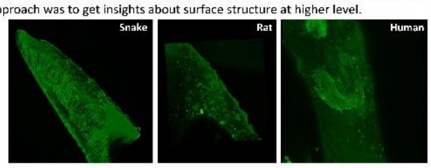
The heatmap presents the normalized values of texture features for each species. The ranking of the values was:

- **Contrast:** human < rat < snake.
- **Correlation:** snake < rat < human.
- **Energy:** snake < rat < human.
- **Homogeneity**: snake < rat < human.

From the texture features analysis it can be observed that human teeth features values indicate a rather smooth, uniform and uncomplex structure. In contrast, snake tooth texture features indicate a complex and less predictable structure. Rat tooth showed values between human and snake, indicating an intermediate smoothness.

3D images:

Besides AFM experiments allowed to get information about the microtopography. LSCM was used to obtain 3D images. The images from rat and snake teeth were taken in the tip part. Human tooth was smoother and thicker, making imaging hard. For this sample, the image was taken in a scratch of the surface. The aim of this approach was to get insights about surface structure at higher level.



The images showed that snake tooth surface has an undulated regular pattern. Rat tooth has an irregular surface with some attached particles. Human, in contrast, has a smooth surface that only can be imaged in scratched.

Acknowledgements

Profesor: Narine Sarvazyan PhD. Instructors: Gevorg Ghukasyan, Daniel Polianczyk and Sergei Fedotov. We acknowledge to Arsen Kishmiryan for kindly provide us with gyurza teeth and Ekaterina Baidiuk for her support with LSCM experiments.

References

[1] https://bushguide101.com/snake-teeth-and-fangs, [2] https://www.petmd.com/exotic/how-care-rat-teeth, [3] https://en.wikipedia.org/wiki/Human_tooth, [4] https://bio-see.net/afm, [5] https://bio-see.net/hyperspectral, [6] https://bio-see.net/confocal, [7] Abdel Gawad AL et al. "Classification of Dental Diseases Using Hyperspectral Imaging and Laser Induced Fluorescence", *Photodiagnosis and Photodynamic Therapy* (2018).

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Comparative Study of Butterfly Wings

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Dr. Narine Sarvazyan
Dr. Sergey Fedotov
Dr. Daniel Polianczyk
Dr. Gevorg Ghukasyan

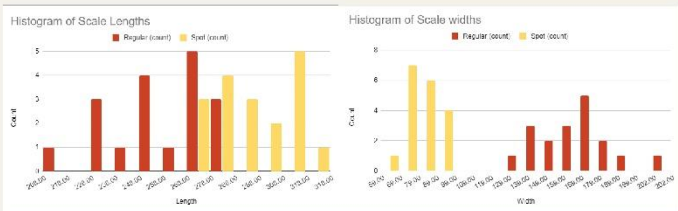
Nazeli Ter-Petrosyan, Arbi Balaban, Aram Adamyan

Abstract

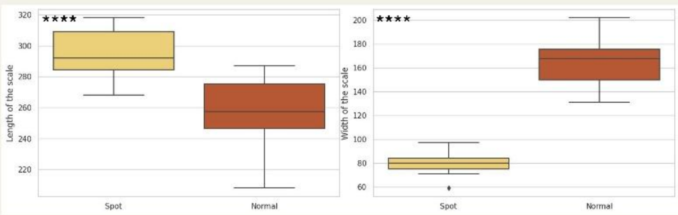
This study utilized three distinct microscopy techniques to examine the scales of two butterfly species, Colias alfacariensis (BFY, left) and Issoria lathonia (QS, right). QS displayed highly fluorescent, metallic spots, setting it apart from its counterpart, and BFY was characterized by an abundance of hair-like scales.

Moreover, the observations showed that the scales located on the spot of BFY were structurally distinct from the regular scales. This study provides valuable insights into the microscopic architecture of butterfly scales and their varying optical properties.

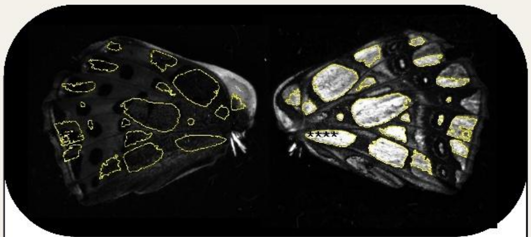
Butterfly wings are covered with tiny scales that give them their color pattern, helping them to avoid predators, regulate temperature, and attract mates.

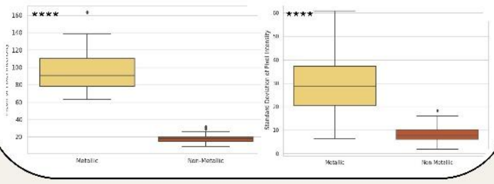


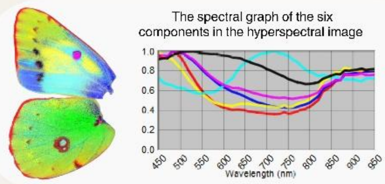
The examination of BFY's spot and regular scales revealed differences in both length and width. The T-test analysis indicated a distinct and significant separation, supported by low p-values.

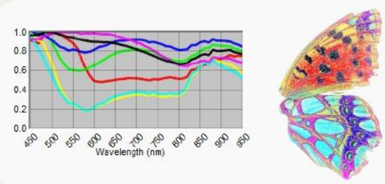


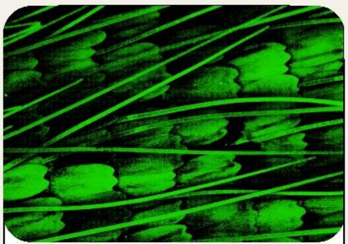
QS's metallic scales are highly autofluorescent compared to the regular scales. The average autofluorescence intensity and standard deviation measured from the highlighted regions show the notable difference.



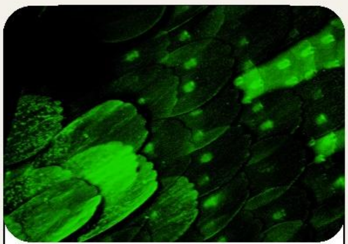






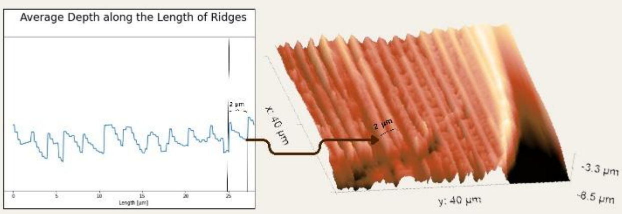


Confocal microscopy image of the hair-like scales on BFY's wings



Confocal microscopy image of the shiny and transparent scales of QS's wings

Image processing techniques were used to calculate the average amplitude of each ridge and to estimate the length between the ridges of the scale



Future work

Understanding the butterfly wings' structure and properties can enable scientists to develop new materials. Inspired by the nanostructures of the scales, the engineers have created various innovations, including plasmonic paints, water resistance, and self-cleaning surfaces that have been used in fashion, aerospace, and construction. In the upcoming stages, researchers can make significant advancements by investigating the thermoregulatory, electromagnetic, and adhesive characteristics of the scales.

Conclusion

In conclusion, the acquired images and analysis revealed clear differences in the structure of butterfly scales. QS has bright, metallic scales, while BFY doesn't. Unlike QS, BFY has hair-like scales on its wings. Lastly, the statistical analysis demonstrated that BFY's spot scales are structurally distinct from the regular wing scales and that QS's metallic scales have significantly more autofluorescence than normal ones.

Comparative Analysis of Brewer's and Baking Yeast:

Insights from AFM, Confocal, and Hyperspectral Imaging

By: Hayk Hovhannisyan | Kristina Ghahramanyan | Kamo Aghbalyan

Introduction

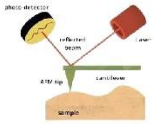
The yeast used in bread and beer production are made from different strains of the same species, Saccharomyces cerevisiae. **Brewer's** yeast is particularly rich in B vitamins thiamine, riboflavin, niacin, etc, as well as minerals such as chromium and selenium. **Baking** yeast, on the other hand, is rich in folate and contains some trace minerals.

This study explores the variations between these closely related yeast strains. The main goal of this analysis is to understand the extent to which of the slight divergence in the origin has affected yeasts.

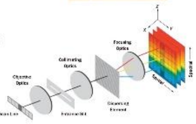
Methodology

To activate the yeast, the **5g** of its granules were submerged in **100 ml** of lukewarm water and mixed with **15g** of sugar. The mixture was then incubated under **40°C for 1 hour.**

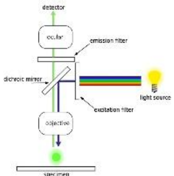
- **For AFM,** the yeast cells were observed by diluting the mixtures and drying them on a cover glass. **Gwyddion** image analysis software was used for the measurements.



- **For Hyperspectral Imaging,** both the granules and cells were used. For bright-field mode, a white sheet of paper was used as a reference. Images were analyzed with the **ImageJ** software.



- **For Confocal Microscopy,** less diluted samples were used, with an addition of a cover slip. Specimens were illuminated with wavelengths of 3 lasers **(488, 543, 633 nm).**
- **Python Numpy** was used to create the autofluorescence intensity graphs.

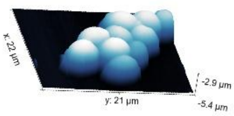


Results

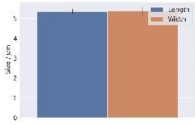
AFM

The baking yeast cells exhibit a predominantly round shape with an aspect ratio close to **1:1**, while brewer's yeast displayed a more elongated shape.

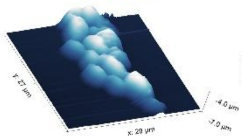
Baking Yeast cells under AFM 3D



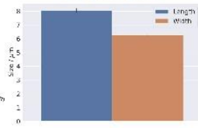
Average Length and Width of Baker strain



Brewer's Yeast cells under AFM 3D

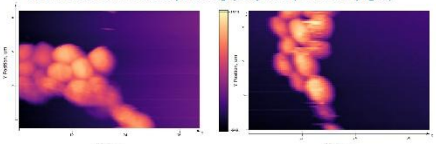


Average Length and Width of Brewers strain



There is a notable size difference between the two yeast cells. Baking yeast is approximately **5.2 ± 0.23 µm** in diameter and brewer's yeast has a length of **8 ± 0.14 µm** and a width of **6.3 ± 0.12 µm.**

Yeast cells under AFM | Baking (left) and (Brewers (right)



Acknowledgements

Professor: Narine Sarvazyan
Instructors: Daniel Polianczyk, Sergey Fedotov, Gevorg Ghukasyan
L.A.Orbeli Institute of Physiology NAS RA

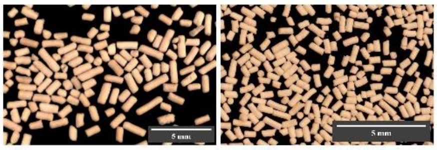
Hypotheses

- **Hypotheses to be revealed by AFM:**
  - There is a difference in the cell shape and size between brewers and baking yeast.
- **Hypotheses to be revealed by HSI:**
  - Differences in the spectral properties of both types of yeasts will allow to distinguish their heterogeneity.
- **Hypotheses to be revealed by Confocal Microscopy:**
  - The levels of autofluorescence of brewer's and baking yeasts are different.

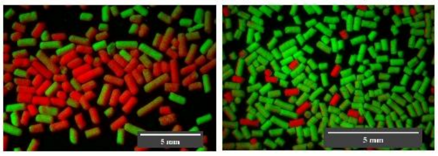
HSI

Hyperspectral imaging confirmed the heterogeneity of both types of yeast granules. **Real Component Analysis and manual unmixing** could differentiate two distinct shades of each granule type. Heterogeneity of baking yeasts, is detectable with human eyes, yet with the brewer's sample, only HSI images allow such results.

RGB image of granules | Baking (left) and Brewers (right)

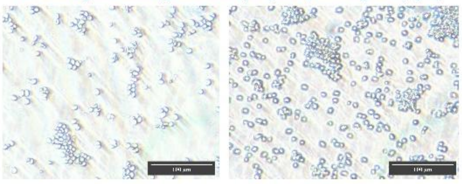


HSI in fluorescent mode | Baking (left) and Brewers (right)

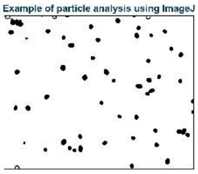


To find out why the brewer's yeast had a few very distinctly differing spectral variations, 2 samples of those yeasts were activated: taking **pseudo-green** and **pseudo-red granules**. Under the same conditions, both subtypes of brewer's yeast displayed differences in the amount of cells. The pseudo-red granules resulted in **fewer active cells**. Over 8 randomly chosen areas (4 for each subtype) of the same size, there were **667** cells for the pseudo-green group and **370** cells for the pseudo-red one.

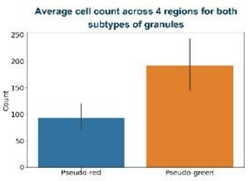
Brewer's yeast cells | pseudo-red (left) and pseudo-green (right)



Example of particle analysis using ImageJ

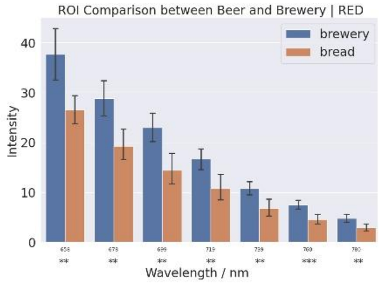


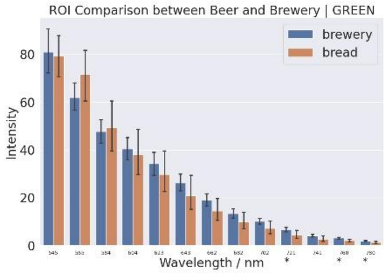
Average cell count across 4 regions for both subtypes of granules

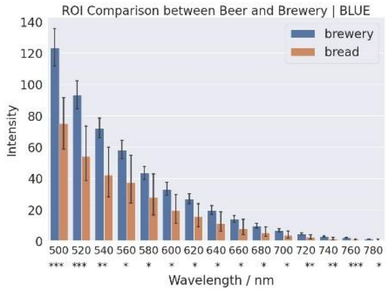


Confocal Microscopy

Utilizing **Leica Lambda Scans**, the autofluorescence intensity of brewer's yeast, characterized by elevated levels of fluorophores such as pyridoxine and riboflavin, was reconstructed. Two samples of each yeast type underwent imaging with three lasers, generating a **total of 12 graphs.** Subsequent removal of noise and graph averaging revealed a discernible elevation in autofluorescence intensity in brewer's yeast compared to the other type under identical wavelengths and laser intensity conditions (**99.9% confidence** ***).

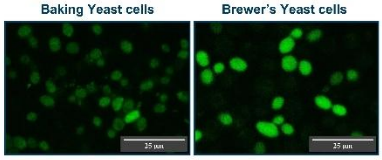






It was observed that the intensity of autofluorescence varied not only based on yeast type but even from cell to cell.

Autofluorescence of Baking Yeast cells | Autofluorescence of Brewer's Yeast cells



Future Studies

Analyzing baking and brewer's yeast using the mentioned methods could have much potential in future studies. Yeast effectiveness is a key factor in various areas of their application. Techniques like AFM can provide detailed examination of yeast-based products on the nano scale, which can help to ensure high quality. Hyperspectral imaging will be useful for measuring the composition and revealing the presence of nutrients in different yeast types. Confocal microscopy is another technique that can track the quality of yeast cells, specifically by adding different stains to the samples.

Conclusion

In conclusion, the in-depth analysis of two types of yeasts utilizing **AFM**, **Confocal**, and **HSI** techniques highlights the unique characteristics of yeasts from their dry form to the cellular level. The analysis shows that different types of yeast cells and their granules exhibit variations in **cell shape, size,** and **autofluorescence** in both yeast samples. Both granules types exhibit heterogeneous spectral properties, even though only one could be distinguished without the use of a Hyperspectral camera. The experiment findings suggest that yeast quality may vary based on slight variations in the spectral properties of their granules. The dissimilarities in the autofluorescence intensity uncover further information about the yeasts. The results can become an indication of possible deviations in metabolic activity and/or uneven concentration of fluorophores in two types of yeasts.

College of Science and Engineering
American University of Armenia | 12 Dec, 2023

Stomata aperture difference in young and old leaves

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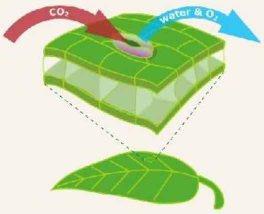
American University of Armenia[1]

Introduction

Stomata are microscopic pores on leaf surfaces crucial in plant physiology, governing vital processes such as gas exchange and water evaporation rate. Therefore, stomatal aperture regulation is crucial to the plant's metabolism. This pilot study employs advanced microscopy techniques to delve into the nuances of stomatal aperture, leaf reflectance spectra, and surface morphology.

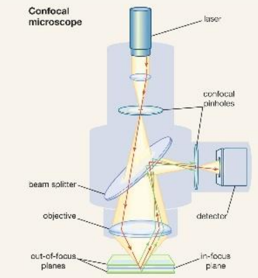


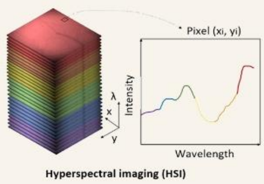
Objectives

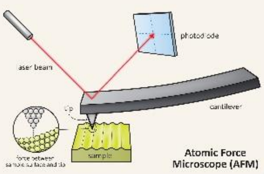
By combining advanced imaging techniques and spectral analysis, our study aims to compare stomata of young and old leaves, and assess differences in their reflectance spectra. We examined a tropical plant and its two species, which we refer to as 1st and 2nd species in this work, they are shown in Figure 1.

- **Stomatal Aperture Analysis:**
  - Utilize Confocal Microscopy to investigate and compare stomatal apertures in old and young leaves.
  - **Hypothesis:** Stomata of the newer leaf should have larger aperture
- **Leaf reflectance spectra comparison:**
  - Employ Hyperspectral Imaging (HSI) to examine the reflectance spectra of leaves, and find regions with a significant difference
- **Atomic Force Microscopy (AFM) for Surface Characterization:**
  - Employ AFM to capture detailed images of stomatal surfaces, enhancing results from previous points.

Figure 1. Examples of the 2 types of leaves used in experiments.







Methodology

Confocal: Young and old leaves are cut into squares of several millimeters in size, mounted between a slide and a coverslip. Both top and bottom sides were imaged using Leica LSCM, with objectives of 20x and 63x magnification. Imaging is conducted based on autofluorescence of stomata. Best results were achieved using combination of 500-590μm and 650-720μm emission filters, as shown in Figure 3.

HSI: Reflectance based Hyperspectral images of young and old leaves put side-by-side are acquired using Nuance HSI camera. Leaves are cut and put between slides to ensure flatness and even illumination. A reference cube of the empty slides is aquired after fixing a broadband white lightsource. In addition to hyperspectral cubes, RGB images of each old / young pair of leaves is acquired. Obtained cubes are processed using Nuance software.

AFM: A transparent peel of leaf epidermis is obtained by scratching the leaf surface with a razor blade. Transparent sample enables reasonable detection of stomata with optical microscope to place the AFM needle in region of interest. The stomata are scanned using vibrating mode and processed with Gwyddion software.

Software used: For the processing and measurements of acquired microscopy images ImageJ and Nuance software is used. For data visualization and statistical testing Python and R programming languages are used respectively.

Results

Confocal: The aperture measurements collected from 50 confocal images support the claim that the mean of stomatal aperture is different in young and old leaves, based on the Pooled t-Test. (see Figure 2).

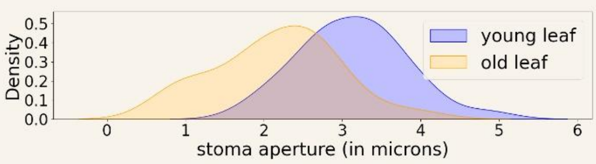


Figure 2. Estimated probability density functions of young and old leaf stomata aperture

$$p - value < 0.01$$

$$\hat{\mu}^{est}_{young} = 3.13,\ \hat{\mu}^{est}_{old} = 2.15$$

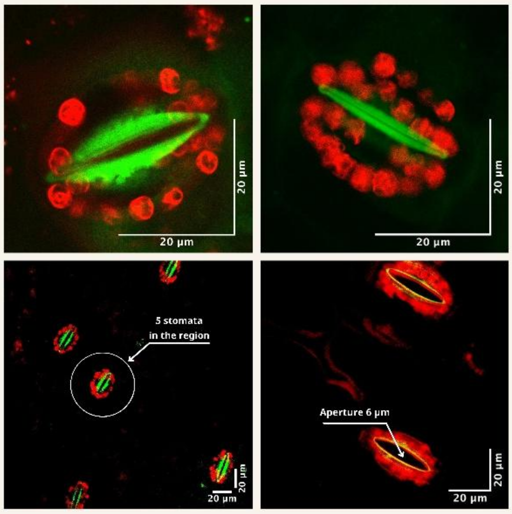


Figure 3. LSCM images of stomata. Overlays 500-590μm and 650-720μm filters. 2nd species

HSI: The analysis of the acquired cubes reveals significant differences in spectra between the young and old leaves of the 1st plant species, in the 650-700μm wavelength region as shown in Figure 4.

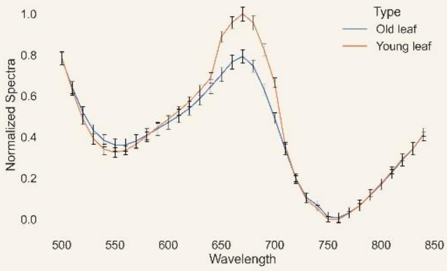
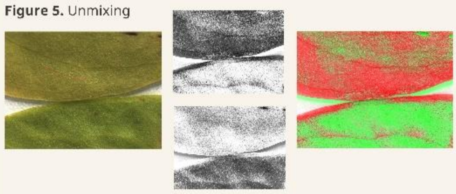


Figure 4. Reflected spectra samples from 5 regions.

Figure 5. Unmixing



The unmixing process is illustrated from left to right Component Images are obtained and combined into a composite unmixed image

AFM: It is observed that the shape of the stoma is not flat from the result of the AFM scanning, as shown in Figure 6.

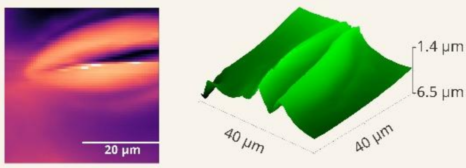


Figure 6. Scanned AFM images

Acknowledgements & Affiliations

We're very thankful to Dr. Sarvazyan for supervising the whole process. We thank Daniel Polianczyk, Sergey Fedotov, and Gevorg Ghukasyan for their enormous help during the experimentation. We thank the Orbeli Institute of Physiology for providing all the necessary equipment and means for conducting the experiments and providing us with samples.

Conclusion

Using LSCM, we observed a statistically significant difference in stomatal aperture in young and old leaves. We also conducted a proof-of-concept study of the leaf reflectance spectra and observed significant differences in the spectra of young and old leaves using HSI.

Stomata regulate the plant's water level, directly linked to its coloring. The methodology of our pilot study can be used in agriculture to assess environmental influence on plants.

Additionally, future studies may include a more detailed examination of stomata using AFM, in particular, to measure the stiffness and electrical properties of guard cells around the stomata of old and young leaves.

UNRAVELING AVIAN MYSTERIES: COMPARATIVE STUDY OF BIRD FEATHERS

Arman Manukyan, Diana Tumasyan, Nina Lazaryan, Vahan Yeranosyan

Acknowledgments

Dr.Narine Sarvazyan
Dr.Sergey Fedotov
Dr.Gevorg Ghukasyan
Dr.Daniel Polianczyk

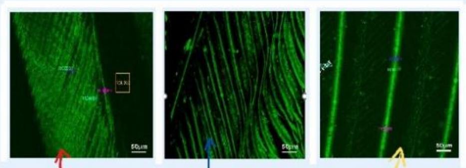
Introduction

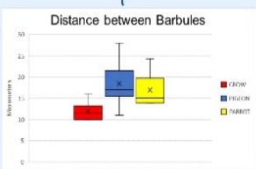
By the use of several advanced microscopy and imaging techniques, the spectral, structural, and morphological characteristics of bird feathers were analyzed. Our sample consisted of feathers from three avian species: *Columba livia* (pigeon - blue color), *Corvus corone* (crow - red color), and *Pyrrhura molinae* (parrot - yellow color). The aim was to examine feather intricacies, including barb and barbule composition, rachis function, and quill morphology. By using microscopy methods, gaining insights into the microscopic architecture of feathers and their diverse optical properties across different avian species was targeted. This research contributes to the broader understanding of avian biology and ecology giving understanding of the relationship between feather structure and function for different species of birds

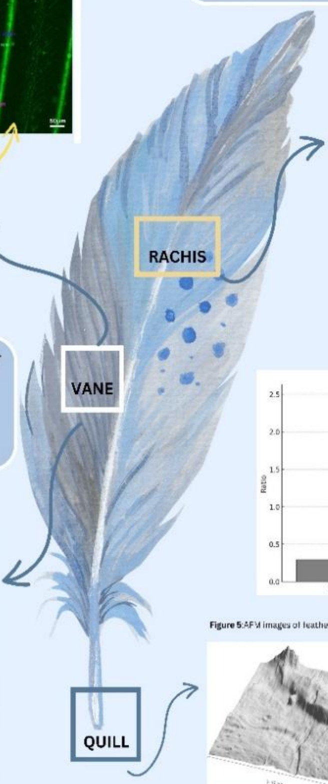
Methodology

- **Atomic Force Microscopy (AFM):** Microscopy technique that utilizes a sharp probe to scan surfaces at the nanoscale, providing high-resolution topographical information and enabling the study of surface properties with exceptional detail. The surface of quills was analyzed to characterize nanostructures using parameters such as surface roughness, adhesion forces, and nanoscale topography. Life Sciences Atomic Force Microscope (LS-AFM) was used for the research.
- **Hyperspectral Imaging (HSI):** Technique that captures detailed spectral information from each pixel in an image, allowing for precise identification and analysis of materials based on their unique spectral signatures. Both **Reflectance** and **Autofluorescence** techniques were employed. HSI AUTO was employed in 420 to 720 wavelength range. HSI REF was employed in 450 to 950 wavelength range, under white light. Nuance Multispectral Imaging System FX was used for the research.
- **Confocal Microscopy:** Microscopy technique that provides high-resolution, three-dimensional images by selectively focusing on a single plane of a specimen while eliminating out-of-focus light. We focused on analyzing the morphology and structure of feathers, including the examination of barbule spacing. Parameters for this analysis included the selection of laser wavelengths, with red lasers chosen over blue and green due to their better performance. Leica confocal laser scanning microscope (2015) was used for the research.

Figure 1 Confocal images under the red laser for all three species (crow - left, pigeon - middle, parrot - right)







Results for Confocal: Confocal microscopy revealed a denser barbule composition in crow feathers compared to pigeons and parrots. The images depict apparent differences that environmental factors and flight patterns could possibly explain. The ANOVA test showed significant results with p-value 0.0094.

Figure 3: HSI Autofluorescence images for feather rachis of all three species (pigeon - upper, crow - middle, parrot - lower)

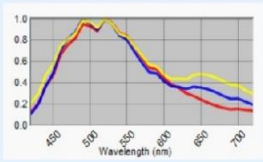
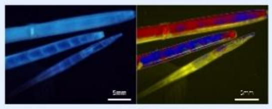
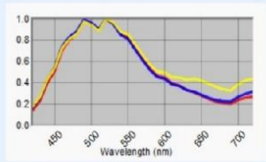
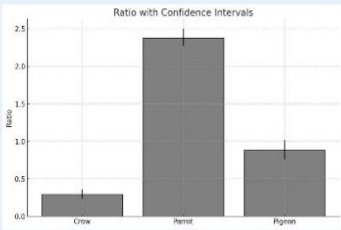


Figure 4: HSI Autofluorescence images for feather quill of all three species (crow - upper, pigeon - middle, parrot - lower)

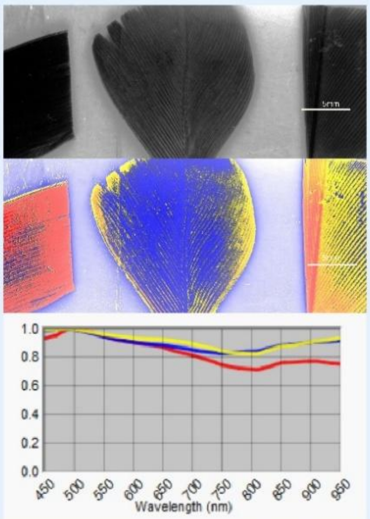






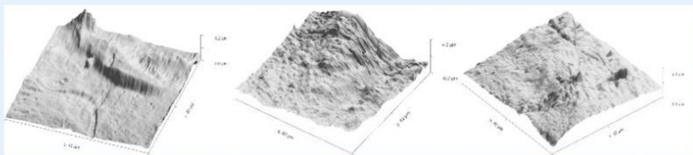
Results for HSI Autoflurescence: While the spectral patterns for all three species appear similar overall, unmixing techniques indicate distinct coloration for the rachis and quills of each species. This suggests subtle yet significant differences in the chemical composition or structural properties of these feather components among the studied avian species. Furthermore, our analysis revealed a pattern between the rachis and vane fluorescence for each species. The ANOVA test showed significant results with p-value of 0.0067.

Figure 2: HSI Reflectance images of the inner part of feather for all three species and their corresponding spectrum (crow - left|red, pigeon - middle|blue, parrot - right|yellow)



Results for HSI Recflectance: The inner parts of feather that look almost identical and not differentiable by eye were examined. Using HSI REF analysis and spectral unmixing technique feathers from visually similar species revealed distinct spectral signatures for each.

Figure 5: AFM images of feather quill for all three species in 40 µm x 40 µm (parrot - left, pigeon - middle, crow - right) and their amplitude



Results for AFM: The surfaces of the quills of our species were examined and some distinct structural differences were discovered. There are varying amplitudes among the species, with parrots exhibiting the least, crows - the greatest, and pigeons falling in between, providing valuable insights into their microscopic characteristics. Referring to this quantification and 3D data, the surface difference is detected among the three birds.

Conclusion

In conclusion, while utilizing the techniques mentioned, it is possible to differentiate the feathers of the mentioned bird species. HSI Auto revealed spectral differences in rachis and species-specific vane-to-rachis intensity ratios; AFM detected surface properties; HSI Ref differentiated the parts of feathers that are visually similar; and Confocal microscopy highlighted varying barbule densities among the birds. These findings collectively underscore the multidimensional nature of avian adaptation and highlight the necessity of employing diverse analytical approaches.

Future work

Performing the research and gathering more data would make the results more reliable. Feathers from different parts of the birds should be examined for future validation. Understanding how these properties vary across different bird species and environmental conditions could offer valuable insights into evolutionary adaptations and ecological resilience. Moreover, having a better understanding of the structure of feathers of different birds could explore the development of novel materials for diverse applications, such as in biomimetic design, aerospace engineering, and textile manufacturing.

A Comparative Study of Red vs Pink Peony Roses

Discoveries from Confocal, AFM, Ref-HSI Imaging modalities

By Margarita Alikhanian, Vahe Petrosyan, Liana Darbinyan, Ara Abovyan

Acknowledgements: Professor: Narine Sarvazyan; Instructors: Daniel Polianczyk, Sergey Fedotov, Gevorg Ghukasyan, L.A. Orbeli Institute of Physiology NAS RA

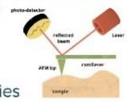
INTRODUCTION

The microscopic analysis of plant structures has opened a new window into understanding the intricate details of their morphology and physiology. In this study, we employed advanced imaging techniques including Confocal Microscopy, Atomic Force Microscopy (AFM), and Hyperspectral Imaging (HSI) with reference (Ref-HSI) mode to compare the microscopic characteristics of petals from two distinct rose species: Rosa rugosa (red rose) and Paeonia lactiflora (peony rose). Roses are not only renowned for their aesthetic appeal but also for their diverse structural characteristics which contribute to their resilience and adaptation. By utilizing these sophisticated imaging techniques, we aimed to elucidate and compare the micro-structural features of these two iconic roses, shedding light on the subtle differences that underlie their distinct morphological and physiological characteristics.

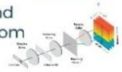
METHODOLOGY

To ensure proper sample preparation, a **1cm** piece was excised from the center of each petal and mounted onto glass slides preserving their natural structure.

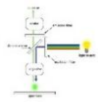
- **For AFM**, The imaging was performed in non-contact mode to obtain high-resolution topographic images of the petal surface with LS-AFM tip-scanning. Gwyddion image analysis software was used for the measurements.



- **For HSI**, Ref-HSI was used to examine the reflection properties of the petal surfaces under 20x, revealing structural details and surface features. Nuance EX Hyperspectral Imaging system from PerkinElmer with 450-950 broadband white light was used for image acquisition.



- **For Confocal**, Images captured were three-dimensional reconstruction of petal tissues at high resolution. Leica Lambda scans ((Leica DMi8) with TCS SPE confocal module (635nm)) were used to reconstruct the autofluorescence intensity under blue light of both Paeonia lactiflora and Rosa rugosa.

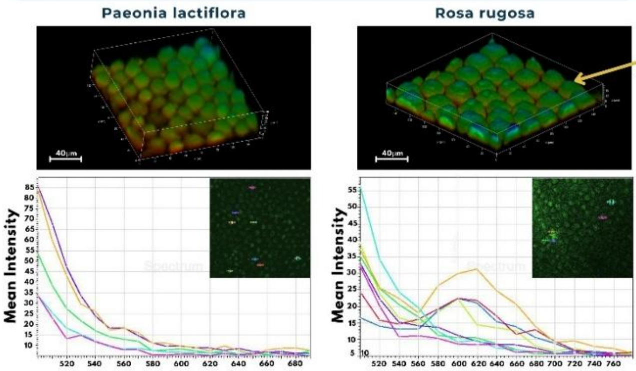


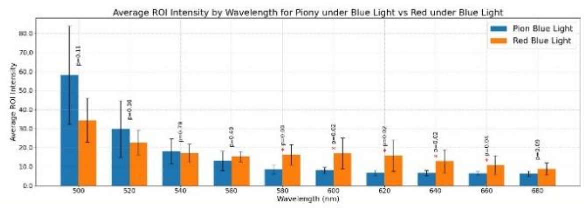
- **Software Used:** For image processing and measurement ImageJ, LasX and Nuance were utilized. Data visualization and statistical analysis were conducted using Python and R programming languages.

RESULTS

CONFOCAL

The petals of both Paeonia lactiflora and Rosa rugosa had conical structures. Analysis across several petals showed that the cones of Rosa rugosa are more evenly distributed across the surface of the petal and have similar height, whereas for Paeonia lactiflora, the cones are distributed unevenly and have dissimilar heights. It was revealed that there is a significant difference between the autofluorecence intensity of the Rosa rugosa and Paeonia lactiflora near 620nm wavelengths (95% confidence).





HYPOTHESIS

Confocal: There is no difference in the structural and spectral properties of a Rosa rugosa and a Paeonia lactiflora.

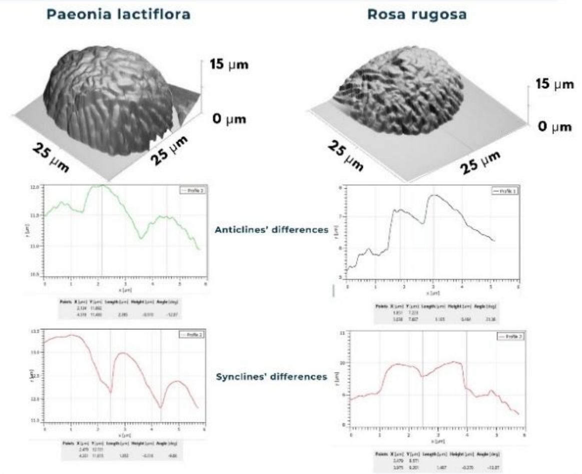
AFM: Rosa rugosa's thicker and bigger petals exhibit higher conical structures compared to Paeonia lactiflora.

HSI: There is no significant difference in the surface reflectance intensity of Rosa rugosa's and Paeonia lactiflora's petals.

RESULTS

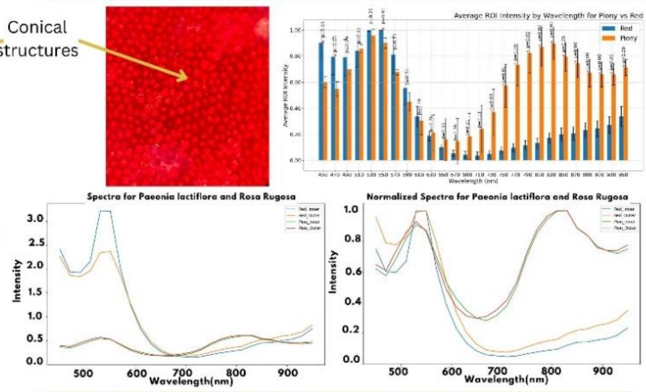
AFM

The petal surfaces of Rosa rugosa exhibited a higher degree of roughness compared to Paeonia lactiflora. Moreover, significant differences were observed in the overall height of surface features, with Paeonia lactiflora petals displaying taller surface structures. Additionally, there were noticeable variations in the surface relief height/depth between the two species.



HSI

The petal surfaces of Paeonia lactiflora and Rosa rugosa were examined under 20x. The reflectance intensity of the petals was acquired from different regions and averaged. Comparative analysis shows that there is a significant difference between the normalized intensity of Paeonia lactiflora and Rosa rugosa especially in wavelengths between 610nm-950nm (99% confidence).



CONCLUSIONS & FUTURE WORK

The advanced microscopic analysis revealed significant differences in the micro-structural characteristics of petals between Rosa rugosa and Paeonia lactiflora. These findings contribute to a better understanding of the morphological and physiological adaptations of these two iconic rose species. Additional findings can be retreived from further analysis.

Study of Armenian Gyurza and Python Molted Skins

Lusine Mheryan, Armen Nalbandyan, Narek Meloyan, Marina Igitkhanian

ACKNOWLEDGMENTS

We're very thankfull to Dr. Sarvazyan, Gevorg Ghukasyan, Daniel Pollanczyk, Sergei Fedotov for their help during the whole process.

Introduction

Snakeskin is a fascinating biological material that plays a crucial role in the survival and adaptation of snakes to their environments. The skin of snakes is a complex structure composed of several layers, each with unique properties and functions. Understanding the structure of snakeskin can provide valuable insights into the biology of these animals. The objective of the study was to analyze and compare the structural properties of the molted skin of two snakes: the Armenian Gyurza and the Python. Additionally, the study aimed to demonstrate the consistency of the obtained results with the known chemical compounds of the molted skin of the considered species of snakes.

Methodology

To analyze the structure of snake skin, we employed several advanced imaging techniques, including Atomic Force Microscopy (AFM), Hyperspectral Imaging (HSI), and Confocal Microscopy. These techniques allowed us to study snake skin's surface morphology, chemical composition, and three-dimensional structure at high resolution.

Confocal Microscopy

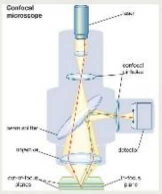


Fig. 1. Confocal Microscope Schematic

The confocal microscope utilizes fluorescence optics and focuses laser light onto a specific spot within the sample, emitting fluorescent light only from that point. A pinhole blocks out-of-focus signals, ensuring clarity. By scanning the specimen in a raster pattern, images of single optical planes are generated, enabling 3D visualization through stacking multiple planes. This technique allows for the analysis of multicolor immunofluorescence using advanced confocal microscopes equipped with multiple lasers and filters. During the research we used blue and green lasers, which enhances the versatility of imaging different fluorophores.

The model of the microscope that was used during the study is Leica confocal laser scanning microscope (2015).

Atomic Force Microscopy

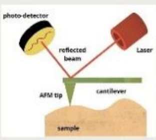


Fig. 2. Atomic Force Microscope Schematic

Atomic force microscopy (AFM) is a type of scanning probe microscopy (SPM) that uses a very sharp probe that is raster-scanned to produce a true 3D topographical map of the surface of a sample with nanoscale resolution.

The model of the microscope that was used during the study is Life Sciences Atomic Force Microscope (LS-AFM).

Hyperspectral Imaging

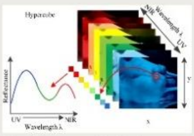


Fig. 3 Hyperspectral Imaging Process

Hyperspectral imaging captures and analyzes the spectral information of an object or material. It involves using a hyperspectral camera to capture across different wavelengths of the electromagnetic spectrum, resulting in a hyperspectral data cube that offers detailed insights into the analyzed object or material. Both Reflectance and Autofluorescence techniques were employed in 420-720 nm wavelength range. The model of the microscope is Nuance Multispectral Imaging System FX.

Results and Findings

Reflectance HSI

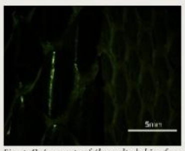


Fig. 4. Outer parts of the molted skins from the back of the snakes. Python(left), Gyurza(right)

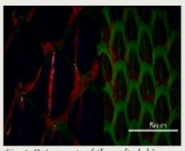


Fig. 5. Outer parts of the molted skins from the back of the snakes - unmixed composites. Python(left). Gyurza(right)

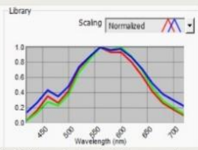


Fig. 6. The spectral graph of the components

Autofluorescence HSI

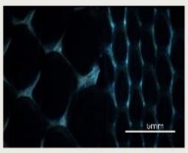


Fig. 7. Outer parts of the molted skins from the back of the snakes. Python(left), Gyurza(right)

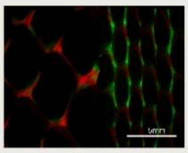


Fig. 8. Outer parts of the molted skins from the back of the snakes - unmixed composites. Python(left). Gyurza(right)

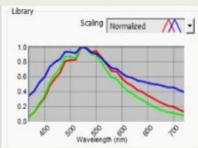


Fig 9. The spectral graph of the components

Confocal Microscopy

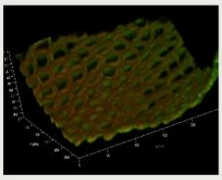


Fig. 10. Gyurza's molted skin, outer part, area of one scale

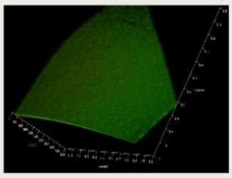


Fig. 11. Python's molted skin, outer part, area of one scale

Atomic Force Microscopy

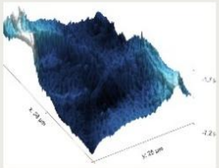


Fig. 12. Gyurza's molted skin, outer part, area of one scale

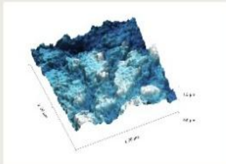


Fig. 13. Python's molted skin, outer part, area of one scale

Chemical Compound of the Skin

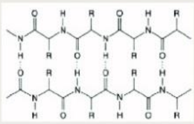


Fig. 14. Chemical structure of beta keratin.

Snake's molted skin comprises mainly beta-type keratin, which does not produce fluorescence. Still, some beta-type keratins have amino acids like **Tryptophan**, **Tyrosine**, and **Phenylalanine** that can fluoresce.

AFM - Confocal

Results from Confocal Microscopy and Atomic Force Microscopy show that the structure of the scales of the two snakes is different. The Python scale has an even distribution, with 'lines' at each height, distributed almost equally around the center of the scale, whereas the Gyurza scales are distributed in circular forms; they do not get one on top of the other and are distributed equally around the skin segment part, around the center line in the back of the snake.

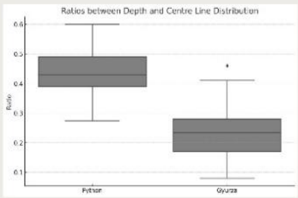


Fig. 15. Ratios between Depth and Centre Line distribution

To quantify those observations, the ratios between the center line of the scale and its depth were calculated for each taken sample. Precisely, ten samples of each species were measured. In Figure 15, the boxplots represent the distributions of the ratios, suggesting a greater value for Python. Additionally, a T-test was conducted to check the hypothesis if the difference in the ratios between the center line of the scale and its depth for two considered species is statistically significant. The hypothesizes of the test are the following:

H0: There is no difference in the ratios of the two species
H1: There is a difference in the ratios of the two species

The p-value of the conducted test equals 0.013, rejecting the hypothesis of no difference between ratios. The results are consistent with the observations from the AFM and Confocal Microscopy.

Conclusion

The comparative analysis found differences in the structure of the molted skin of the Python and Gyurza. The main characteristics of the structure, such as the shape, pattern, size, and depth of the scales, were considered. As a result of the analysis, the molted skin's mechanical properties may be connected with the natural environment in which Gyurzas and Pythons live. Gyurzas inhabit Armenia —a mountainous country—while Pythons come from Eastern Asia, which has flat terrains. The former complicates the movements of the snakes since there are more natural obstacles, thus making the structure more complicated. Additionally, the chemical composition of the molted skin was researched. It was shown that the level of fluorescence was consistent with the types of B-keratins in the composition of the molted skins of the two species.

Effects of dyes on white hair

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Introduction

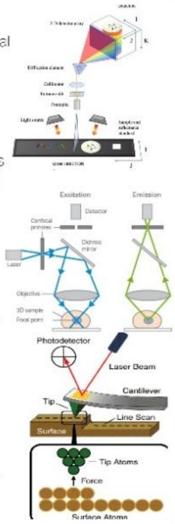
In contemporary society, the practice of hair dyeing is prevalent, reflecting not only aesthetic preferences but also individual expressions of identity. With an increasing number of individuals turning to both natural and chemical hair dyes, understanding the implications of these substances becomes crucial. Our study, grounded in the field of biomedical imaging, seeks to investigate the potential differential effects of natural versus chemical hair dyes on human hair. The primary objective of this research is to meticulously analyze and compare the structural changes, if any, that occur in hair as a result of using these two different types of dyes. Our investigation was structured around a null hypothesis that posits no significant difference between the effects of natural and chemical dyes on hair structure.

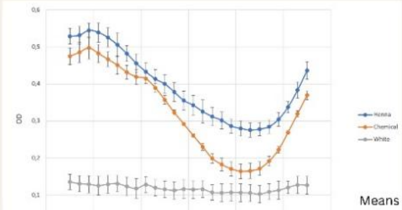
Methods

For this study, white hair from a single individual was sectioned into three parts to minimize variability. One section remained undyed as a control, while the other two were dyed with henna and a chemical dye, respectively, both aiming for a black color. The dyes were applied following the manufacturers' instructions, with each left in the hair for 40 minutes. After dyeing, each section was washed and dried under identical conditions. The effects of the dyes were then analyzed using high-resolution biomedical imaging to compare structural changes in the hair fibers.

Methodology

- Hyperspectral imaging captures data across multiple wavelengths, providing detailed spectral information. Autofluorescence uses natural emission from biological structures after light absorption, aiding in imaging specific cellular compounds without external labels. Combined in an autofluorescence hyperspectral microscope, these technologies allow for precise observation of tissues through their natural fluorescence across various spectral bands, serving as essential benchmarking tools in advancing biomedical imaging. We used the Nuance microscope.
- A confocal microscope uses a coherent laser light source and a pinhole aperture to block out-of-focus light, ensuring that only light from the focal plane reaches the detector. The scanning mechanism systematically moves the laser across the sample, capturing sharp, detailed images at multiple depths by ensuring that reflected light passes through the pinhole. We used the Leica microscope.
- In an AFM, the tip of a cantilever approaches the sample surface closely without making contact. The interaction forces between the tip and the surface cause the cantilever to deflect, which is detected by changes in a laser beam's position reflecting off the cantilever to a photodetector. A piezoelectric scanner adjusts the cantilever's position based on feedback, allowing for highly precise surface scanning. We used the Geyddion software.

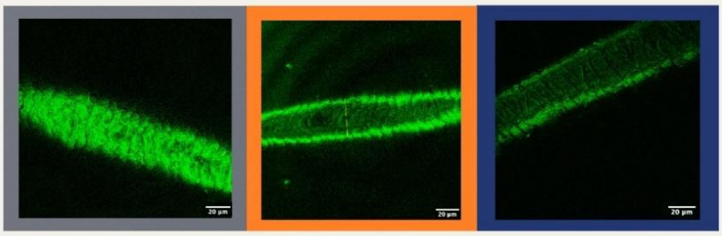




Means of the spectras of 3 samples per type of hair

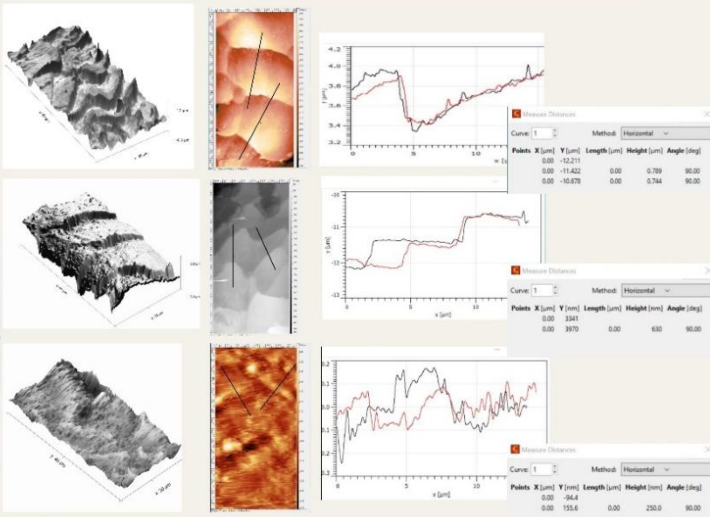
Confocal

Hair dye primarily attaches to the cortex of the hair, which is the inner part of the hair shaft located beneath the cuticle. This is why the cuticle layer in the confocal looks similar to that of white hair: the dye is in the inner part.



AFM

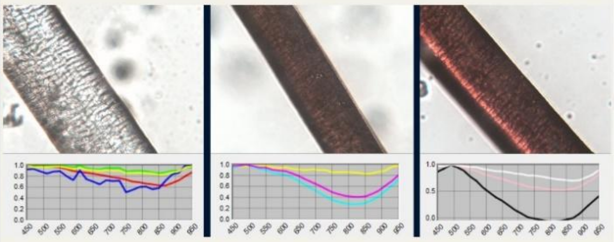
Atomic Force Microscopy (AFM) findings reveal that henna dye appears to "glue" the cuticles of the hair together, resulting in a flatter surface compared to untreated and chemically dyed hair. In contrast, the cuticles in white and chemically-dyed hair remain clearly defined. The henna dye's effect of smoothing the surface and blurring the layers of the hair cuticles explains the common conception that henna dyes make hair shiny. Alsom the integrity of hair cuticles is crucial as they retain moisture and provide elasticity, both essential for maintaining healthy hair.



RESULTS

HSI

As demonstrated in the accompanying images, there are observable differences in the spectral signatures between the henna-dyed hair and the chemically dyed hair. Additionally, a notable visual distinction is evident, highlighted by a streak of light that permeates the henna-dyed hair, further differentiating the effects of the two dye types.



• Grey hair • Chemically dyed hair • Henna dyed hair

The emission spectra of the hair indicate that henna dyes reflect more light, as evidenced by the lower infrared wavelength values for henna-dyed samples. This suggests that henna dyes have enhanced reflective properties in comparison to other dye types.

Conclusion

These studies revealed some interesting facts about hair and hair dyes which were surprising to find out. Seemingly, hair dyes simply give our hair color, but we can see how they also affect the structural integrity and can have lating effects on the overall health. According to the study, henna dyes give hair a natural shine as they smooth the texture of the hair and make the cuticles stick together. They also help with hair strength and thickness.