Tools and Techniques in Biology

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Dr. Ashwani Kumar Dubey

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Preface

The study of various aspects of life activities requires the use of certain techniques and analytical instruments. Physical and chemical sciences have helped scientists in developing some tools and techniques with the help of which biologists have been able to get a clearer insight into the life processes.

Cells in culture or in vitro are a useful model for studying the activity of cells in the whole organism or in vivo. Ten years ago or so cell culture techniques were considered somewhat esoteric. Today because of our better understanding of cell nutrition, metabolism and general growth environment it has become a fairly routine procedure.

Optical or light microscopy involves passing visible light transmitted through or reflected from the sample through a single or multiple lenses to allow a magnified view of the sample.

A colorimeter is a device used in colorimetry. In scientific fields the word generally refers to the device that measures the absorbance of particular wavelengths of light by a specific solution. This device is most commonly used to determine the concentration of a known solute in a given solution by the application of the Beer-Lambert law, which states that the concentration of a solute is proportional to the absorbance.

Atomic spectroscopy is thought to be the oldest instrumental method for the determination of elements. These techniques are introduced in the mid of 19th Century during which Bunsen and Kirchhoff showed that the radiation emitted from the flames depends on the characteristic element present in the flame. The potential of atomic spectroscopy in both the qualitative as well as quantitative analysis were then well established.

Bacteria will grow on practically any source of organic food which provides carbon compounds to be respired for energy, and nitrogen compounds to be

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incorporated into proteins for growth. These substances are normally provided dissolved in water. However, in nature, bacteria can break down solid and insoluble substances by releasing enzymes into the substrate in which they are growing. These substances are thus broken down or digested to simpler substances and the process is called extracellular digestion because it takes place outside the bacterial cells.

Data presentation and analysis forms an integral part of all academic studies, commercial, industrial and marketing activities as well as professional practices. Presentation of data requires skills and understanding of data. It is necessary to make use of collected data which is considered to be raw data. This raw data must be processed to be used or for any application. Data analysis helps in the interpretation of data and help take a decision or answer the research question. This can be done by using various Data processing tools and Softwares.

Cryo-techniques aim to rapidly fix specimens to preserve them as close to their physiological state as possible. Samples are either rapidly frozen generating a frozen hydrated sample for imaging or further processing or may be chemically fixed and cryo-protected before freezing to avoid damage caused by ice crystal formation. In addition to avoiding potential artefacts associated with some other forms of sample preparation, cryo-preserved samples exhibit the best antigenicity, essential for successful immuno-EM.

Immunoblotting (western blotting) is used to identify specific antigens recognized by polyclonal or monoclonal antibodies. This unit provides protocols for all steps starting with solubilization of the protein samples, usually with SDS and reducing agents. Following solubilization, the material is separated by SDS-PAGE and the antigens are electrophoretically transferred to a membrane, a process that can be monitored by reversible staining or Ponceau S staining. The transferred proteins are bound to the surface of the membrane, providing access to immunodetection reagents. After nonspecific binding sites are blocked, the membrane is probed with the primary antibody and washed. The antibody-antigen complexes are tagged with horseradish peroxidase or alkaline phosphatase coupled to a secondary anti-IgG antibody, and detected using appropriate chromogenic or luminescent substrates. Finally, membranes may be stripped and reprobed.

The book covers basic and applied aspects of the subject.

—Dr. Ashwani Kumar Dubey

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Principle and Application of Biology

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MICROSCOPY: PRINCIPLE AND APPLICATION

MICROSCOPY IMAGING

Microscopes have been used since the 1670s to observe cells. Today, microscopes have become indispensable tools in cell biology. There are many more microscopy techniques today that have allowed for better viewing of cells.

In recent years, the world of microscopy has experienced advancements in imaging technologies enabling increased amounts of information for microscopic analysis.

Some of the most common techniques used in cell biology include:

- Bright field microscopy
- Fluorescence microscopy
- Immunofluorescence
- Time lapse microscopy

STAINING

Staining goes hand in hand with microscopy. Although it may be regarded as an important part of microscopy, staining is itself very useful in cell biology. It allows for increased contrast which in turn allows for scientists to view different parts of a cell clearly.

Although staining is highly useful when it comes to viewing specimen under the microscope, it cannot be used when a scientist wants to observe living cells.

Microscopy is the technical field of using microscopes to view samples and objects that cannot be seen with the unaided eye (objects that are not within the resolution range of the normal eye). There are three well-known branches of microscopy: optical, electron, and scanning probe microscopy.

Optical and electron microscopy involve the diffraction, reflection, or refraction of electromagnetic radiation/electron beams interacting with the specimen, and the subsequent collection of this scattered radiation or another signal in order to create an image.

This process may be carried out by wide-field irradiation of the sample (for example standard light microscopy and transmission electron microscopy) or by scanning of a fine beam over the sample (for example confocal laser scanning microscopy and scanning electron microscopy).

Scanning probe microscopy involves the interaction of a scanning probe with the surface of the object of interest. The development of microscopy revolutionized biology and remains an essential technique in the life and physical sciences.

OPTICAL MICROSCOPY

Optical or light microscopy involves passing visible light transmitted through or reflected from the sample through a single or multiple lenses to allow a magnified view of the sample.



Stereo microscope

The resulting image can be detected directly by the eye, imaged on a photographic plate or captured digitally. The single lens with its attachments, or the system of lenses and imaging equipment, along with the appropriate lighting

equipment, sample stage and support, makes up the basic light microscope. The most recent development is the digital microscope, which uses a CCD camera to focus on the exhibit of interest. The image is shown on a computer screen, so eye-pieces are unnecessary.

LIMITATIONS

Limitations of standard optical microscopy (bright field microscopy) lie in three areas;

- The technique can only image dark or strongly refracting objects effectively.
- Diffraction limits resolution to approximately 0.2 micrometres.
- Out of focus light from points outside the focal plane reduces image clarity.

Live cells in particular generally lack sufficient contrast to be studied successfully, internal structures of the cell are colourless and transparent. The most common way to increase contrast is to stain the different structures with selective dyes, but this involves killing and fixing the sample. Staining may also introduce artifacts, apparent structural details that are caused by the processing of the specimen and are thus not a legitimate feature of the specimen.

These limitations have all been overcome to some extent by specific microscopy techniques that can non-invasively increase the contrast of the image.

In general, these techniques make use of differences in the refractive index of cell structures. It is comparable to looking through a glass window: you (bright field microscopy) don't see the glass but merely the dirt on the glass. There is however a difference as glass is a denser material, and this creates a difference in phase of the light passing through. The human eye is not sensitive to this difference in phase but clever optical solutions have been thought out to change this difference in phase into a difference in amplitude (light intensity).

TECHNIQUES

In order to improve specimen contrast or highlight certain structures in a sample special techniques must be used. A huge selection of microscopy techniques are available to increase contrast or label a sample.

- Four examples of transilumination techniques used to generate contrast in a sample of tissue paper. 1.559 ìm/pixel.

Bright field illumination, sample contrast comes from absorbance of light in the sample.

Cross-polarized light illumination, sample contrast comes from rotation of polarized light through the sample. Dark field illumination, sample contrast comes from light scattered by the sample.

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Phase contrast illumination, sample contrast comes from interference of different path lengths of light through the sample.

BRIGHT FIELD

Bright field microscopy is the simplest of all the light microscopy techniques. Sample illumination is via transmitted white light, i.e. illuminated from below and observed from above. Limitations include low contrast of most biological samples and low apparent resolution due to the blur of out of focus material. The simplicity of the technique and the minimal sample preparation required are significant advantages.

OBLIQUE ILLUMINATION

The use of oblique (from the side) illumination gives the image a 3-dimensional appearance and can highlight otherwise invisible features. A more recent technique based on this method is *Hoffmann's modulation contrast*, a system found on inverted microscopes for use in cell culture. Though Oblique illumination suffers from the same limitations as bright field microscopy (low contrast of many biological samples; low apparent resolution due to out of focus objects), it may highlight otherwise invisible structures.

DARK FIELD

Dark field microscopy is a technique for improving the contrast of unstained, transparent specimens. Dark field illumination uses a carefully aligned light source to minimize the quantity of directly-transmitted (unscattered) light entering the image plane, collecting only the light scattered by the sample. Dark field can dramatically improve image contrast – especially of transparent objects – while requiring little equipment setup or sample preparation. However, the technique suffers from low light intensity in final image of many biological samples, and continues to be affected by low apparent resolution.

Rheinberg illumination is a special variant of dark field illumination in which transparent, coloured filters are inserted just before the condenser so that light rays at high aperture are differently coloured than those at low aperture (i.e. the background to the specimen may be blue while the object appears self-luminous red). Other color combinations are possible but their effectiveness is quite variable.

DISPERSION STAINING

Dispersion staining is an optical technique that results in a coloured image of a colorless object. This is an optical staining technique and requires no stains or dyes to produce a color effect. There are five different microscope configurations

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used in the broader technique of dispersion staining. They include brightfield Becke line, oblique, darkfield, phase contrast, and objective stop dispersion staining.

PHASE CONTRAST

More sophisticated techniques will show proportional differences in optical density. Phase contrast is a widely used technique that shows differences in refractive index as difference in contrast. It was developed by the Dutch physicist Frits Zernike in the 1930s (for which he was awarded the Nobel Prize in 1953). The nucleus in a cell for example will show up darkly against the surrounding cytoplasm. Contrast is excellent; however it is not for use with thick objects. Frequently, a halo is formed even around small objects, which obscures detail. The system consists of a circular annulus in the condenser, which produces a cone of light.

This cone is superimposed on a similar sized ring within the phase-objective. Every objective has a different size ring, so for every objective another condenser setting has to be chosen. The ring in the objective has special optical properties: it, first of all, reduces the direct light in intensity, but more importantly, it creates an artificial phase difference of about a quarter wavelength. As the physical properties of this direct light have changed, interference with the diffracted light occurs, resulting in the phase contrast image. One disadvantge of phase-contrast microscopy is halo formation (halo-light ring).

DIFFERENTIAL INTERFERENCE CONTRAST

Superior and much more expensive is the use of interference contrast. Differences in optical density will show up as differences in relief. A nucleus within a cell will actually show up as a globule in the most often used differential interference contrast system according to Georges Nomarski. However, it has to be kept in mind that this is an *optical effect*, and the relief does not necessarily resemble the true shape. Contrast is very good and the condenser aperture can be used fully open, thereby reducing the depth of field and maximizing resolution.

The system consists of a special prism (Nomarski prism, Wollaston prism) in the condenser that splits light in an ordinary and an extraordinary beam. The spatial difference between the two beams is minimal (less than the maximum resolution of the objective). After passage through the specimen, the beams are reunited by a similar prism in the objective.

In a homogeneous specimen, there is no difference between the two beams, and no contrast is being generated. However, near a refractive boundary (say a nucleus within the cytoplasm), the difference between the ordinary and the extraordinary beam will generate a relief in the image. Differential interference

contrast requires a polarized light source to function; two polarizing filters have to be fitted in the light path, one below the condenser (the polarizer), and the other above the objective (the analyzer). Note: In cases where the optical design of a microscope produces an appreciable lateral separation of the two beams we have the case of classical interference microscopy, which does not result in relief images, but can nevertheless be used for the quantitative determination of mass-thicknesses of microscopic objects.

INTERFERENCE REFLECTION MICROSCOPY

An additional technique using interference is interference reflection microscopy (also known as reflected interference contrast, or RIC). It is used to examine the adhesion of cells to a glass surface, using polarized light of a narrow range of wavelengths to be reflected whenever there is an interface between two substances with different refractive indices. Whenever a cell is attached to the glass surface, reflected light from the glass and that from the attached cell will interfere. If there is no cell attached to the glass, there will be no interference.

Interference reflection microscopy can be obtained by using the same elements used by DIC, but without the prisms. Also, the light that is being detected is reflected and not transmitted as it is when DIC is employed.

DECONVOLUTION

Fluorescence microscopy is a powerful technique to show specifically labeled structures within a complex environment and to provide three-dimensional information of biological structures. However, this information is blurred by the fact that, upon illumination, all fluorescently labeled structures emit light, irrespective of whether they are in focus or not. So an image of a certain structure is always blurred by the contribution of light from structures that are out of focus. This phenomenon results in a loss of contrast especially when using objectives with a high resolving power, typically oil immersion objectives with a high numerical aperture.

However, blurring is not caused by random processes, such as light scattering, but can be well defined by the optical properties of the image formation in the microscope imaging system. If one considers a small fluorescent light source (essentially a bright spot), light coming from this spot spreads out the further out of focus one is.

Under ideal conditions, this produces an “hourglass” shape of this point source in the third (axial) dimension. This shape is called the point spread function (PSF) of the microscope imaging system. Since any fluorescence image is made up of a large number of such small fluorescent light sources, the image is said to be “convolved by the point spread function”.

Knowing this point spread function means that it is possible to reverse this process to a certain extent by computer-based methods commonly known as deconvolution microscopy. There are various algorithms available for 2D or 3D deconvolution.

They can be roughly classified in *nonrestorative* and *restorative* methods. While the nonrestorative methods can improve contrast by removing out-of-focus light from focal planes, only the restorative methods can actually reassign light to its proper place of origin. This can be an advantage over other types of 3D microscopy such as confocal microscopy, because light is not thrown away but reused. For 3D deconvolution, one typically provides a series of images derived from different focal planes (called a Z-stack) plus the knowledge of the PSF, which can be derived either experimentally or theoretically from knowing all contributing parameters of the microscope.

SUB-DIFFRACTION TECHNIQUES

A multitude of super-resolution microscopy techniques has been developed in recent times which circumvent the diffraction barrier.

This is mostly achieved by imaging a sufficiently static sample multiple times and either modifying the excitation light or observing stochastic changes in the image.

Knowledge of and chemical control over fluorophore photophysics is at the core of these techniques, by which resolutions of ~20 nanometers are regularly obtained.

SERIAL TIME-ENCODED AMPLIFIED MICROSCOPY

Serial time encoded amplified microscopy (STEAM) is an imaging method that provides ultrafast shutter speed and frame rate, by using optical image amplification to circumvent the fundamental trade-off between sensitivity and speed, and a single-pixel photodetector to eliminate the need for a detector array and readout time limitations The method is at least 1000 times faster than the state-of-the-art CCD and CMOS cameras.

Consequently, it is potentially useful for a broad range of scientific, industrial, and biomedical applications that require high image acquisition rates, including real-time diagnosis and evaluation of shockwaves, microfluidics, MEMS, and laser surgery.

EXTENSIONS

Most modern instruments provide simple solutions for micro-photography and image recording electronically. However such capabilities are not always

present and the more experienced microscopist will, in many cases, still prefer a hand drawn image to a photograph. This is because a microscopist with knowledge of the subject can accurately convert a three dimensional image into a precise two dimensional drawing. In a photograph or other image capture system however, only one thin plane is ever in good focus. The creation of careful and accurate micrographs requires a microscopical technique using a monocular eyepiece. It is essential that both eyes are open and that the eye that is not observing down the microscope is instead concentrated on a sheet of paper on the bench besides the microscope. With practice, and without moving the head or eyes, it is possible to accurately record the observed details by tracing round the observed shapes by simultaneously "seeing" the pencil point in the microscopical image.

Practicing this technique also establishes good general microscopical technique. It is always less tiring to observe with the microscope focused so that the image is seen at infinity and with both eyes open at all times.

LIGHT MICROSCOPE AND PHASE CONTRAST MICROSCOPE

Light microscopes date at least to 1595, when Zacharias Jansen (1580–1638) of Holland invented a compound light microscope, one that used two lenses, with the second lens further magnifying the image produced by the first. His microscopes were collapsing tubes used like a telescope in reverse, and produced magnifications up to nine times (9x).

Antony van Leeuwenhoek (1632–1723) invented a simple (one-lens) microscope around 1670 that magnified up to 200x and achieved twice the resolution of the best compound microscopes of his day, mainly because he crafted better lenses. While others were making lenses by such methods as squashing molten glass between pieces of wood, Leeuwenhoek made them by carefully grinding and polishing solid glass. He thus became the first to see individual cells, including bacteria, protozoans, muscle cells, and sperm.

Englishman Robert Hooke (1635–1703) further refined the compound microscope, adding such features as a stage to hold the specimen, an illuminator, and coarse and fine focus controls. Until 1800, compound microscopes designed by Hooke and others were limited to magnifications of 30x to 50x, and their images exhibited blurry edges (spherical aberration) and rainbowlike distortions (chromatic aberration). The most significant improvement in microscope optics was achieved in the nineteenth century, when business partners Carl Zeiss (1816–1888) and Ernst Abbe (1840–1905) added the substage condenser and developed superior lenses that greatly reduced chromatic and spherical aberration, while permitting vastly improved resolution and higher magnification.