Optical, Confocal, and 4Pi Microscopy

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Introduction

Optical microscopy has been in existence for many centuries. Since the viewing of the first cell, man has continually attempted to view smaller and smaller objects. In 1873, Ernst Abbe first discovered that diffraction limited the resolution of the optical microscope. And even with the advances in confocal and multiphoton microscopes, these limitations have never been lifted.

However, in recent years, developments in microscopy are on the rise. The stimulated emission depletion microscopy has been able to resolve to 28 nm (Hell). And even more developments in the areas of nanoscale imaging are on the rise.

The purpose of this paper is to give an overview of the optical microscope with its virtues and shortcomings and to describe the issues of lateral and axial resolution. Afterwards, it will be shown how confocal microscopy improves the resolution in optical microscopy and how 4Pi microscopy works.

Optical Microscopy

A Brief History of the Optical Microscope

The optical microscope is the oldest of all the microscopes and one of the pinnacles of modern invention; however, its inventor still remains largely unknown. Those credited with its invention include Dutch spectacle-makers, Hans and Zacharias Janssen, and Italian astronomer, and lens-maker, Galileo Galilei (Wikipedia). Galileo’s compound microscope, a microscope invented in 1609 with one concave lens and one convex lens, was the first device to be called a microscope. Christian Huygens in the late 1600s improved on the design of the microscope with a simple two lens ocular system that was achromatically corrected. Anton van Leeuwenhoek was the first to use his homemade microscopes to study microorganisms, thus bringing the microscope to the attention of biologists.

Components

All optical microscopes have the same four basic components: the eyepiece, the objective lens, the stage and the illumination source (Wikipedia). The eyepiece is used to bring the image into focus for viewing. It is usually a cylinder containing two or more lenses. In high performance microscopes, the objective lens and eyepiece are matched to provide the best optical
performance. The objective lens is another cylinder containing one or more lenses. These lenses are used to collect light from the sample. Typically, a standard compound microscope will have three objective lenses: a scanning lens, a low power lens, and a high power lens. Advanced microscopes have an additional lens called an oil immersion lens. The stage is placed below the objective lens and holds the sample. In the center of the stage is a hole through which light passes and enters the sample. Lastly, the illumination source is below the stage and controllable through an optical device called a condenser.

Figure 1. Basic Optical Microscope (Free Info Society)

More modern microscopes have other features such as transmission illumination, phase contrast microscopy and differential interference contrast microscopy, digital cameras (Wikipedia).

**Optics**

In a microscope (see Figure 2 below), the objective lens is placed close to the sample so that the light comes into focus inside the microscope tube, creating an enlarged inverted image of the specimen (Wikipedia). The eyepiece, a compound lens with one lens near the front of the eyepiece and the other near the back forming an air-separated couplet, then focuses the rays.
Often, the virtual image will focus between the two eyepiece lenses. The first lens will focus the real image and the second image will focus the virtual image for the eye.

Figure 2. Optical Path in a Standard Compound Microscope (Hecht 214)
**Lateral and Axial Resolution**

Resolution may be given by the following equation shown in the figure below, with $\lambda$ representing the wavelength of the light and NA representing the numerical aperture (the light gathering capabilities of a lens, $n\sin \alpha$, with $n =$ index of refraction and $\alpha$ the angle of refraction) (Rack).

$$R = \frac{(0.61 \cdot \lambda)}{N.A.}$$

*Figure 3. Resolution Equation (Rack)*

Therefore, it is easy to see that the wavelength of visible light, being between 400 and 700 nm limits the resolution of the optical microscope. The best optical microscopes have only been able to obtain a focal resolution of 180 nm and a optical axis resolution of 500 nm (Hell).

**Advantages and Disadvantages**

Besides the limiting resolution discussed above, the depth of field of an optical microscope is hindered again by the wavelength restriction (see figure below) (Rack). The variables are the same as those defined above.

$$DOF \approx \frac{\lambda}{N.A.}$$

*Figure 4. Depth of Field Equation (Rack)*

When comparing between an optical microscope and a scanning electron microscope (SEM) as in the figure below, it is clear why optical microscopes are not always the preferred choice.
Despite these limitations, optical microscopy is attractive for numerous reasons. The chief attraction is the ease of use (ACEPT W^3 Group). Optical microscopy has been in use so long that it does not take much expertise to understand how to use. Another attractive quality is the fact that the optical microscope may be used to view samples that are in either air or water. And the images provided through optical microscopy are in real color instead of virtual colors typical in other forms of microscopy.

**Confocal Microscopy**

To combat the inherent problems with the optical microscope, the confocal microscope was developed. First patented in 1957 by Marvin Minsky, the confocal microscope has most of the same primary parts as the optical microscope: the objective lens, eyepiece, and stagepiece (Wikipedia). The primary difference between the confocal microscope and the optical microscope lies in the illumination.

In a confocal microscope, there are two additional components (see figure below): the laser illumination source and a pinhole aperture (Paddock). Instead of flooding the sample with an illumination source, a focused beam of light is used to illuminate the sample. The light then is focused as in the optical microscope by the objective lens. However, before reaching the eyepiece, the out-of-focus rays are filtered out using a pinhole detector. This greatly improves the quality of the image.
Even with the improvements over the optical microscope, the confocal microscope (see figure below) still does not rectify all the problems. Confocal microscopy improves the quality of the three dimensional image but makes little improvement on the resolution (Hell). Furthermore, the confocal microscope can only take an image of a very small portion of the sample, requiring the microscope to scan across the surface.
4Pi Microscopy

An emergent optical microscopy, 4Pi microscopy, involves using two high-aperture lenses to increase the viewing resolution (Lakowicz 393). In optical and confocal microscopy, the resolution is also defined by the objective lens. In 4Pi microscopy, two objective lenses are used to provide a larger aperture. The wavelengths then interfere constructively, improving the quality of the image. Although the full 4Pi is inaccessible (see figure below), the name is retained as a reminder of the principles.
Besides providing a clearer image, 4Pi microscopy only requires approximately one half of the light to view the image (Hell & Stelzer). This provides protection against out-of-focus light rays and boosts the signal.

**Conclusions**

Optical microscopy is a valuable method that is used in many areas of expertise. It is invaluable in the biological fields and in metallurgy. Improvements in optical microscopy, including 4Pi microscopy, are finally breaking the diffraction limitation, allowing closer than ever views of samples that would previously been imaged using SEM. The future promises even more advances in this field with the refinement of 4Pi microscopy to even finer resolutions.
Bibliography


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