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Clinical Microscopy: Performance, Maintenance and Laser Safety

Tae Bok Lee

Confocal Core Facility, Center for Medical Innovation, Seoul National University Hospital, Seoul, Korea

임상에서의 현미경: 작동, 유지보수 및 레이저 안전

이태복

서울대학교병원 의학연구혁신센터 공초점현미경실

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A microscope is the fundamental research and diagnostic apparatus for clinical investigation of signaling transduction, morphological changes and physiological tracking of cells and intact tissues from patients in the biomedical laboratory science. Proper use, care and maintenance of microscope with comprehensive understanding in mechanism are fully requested for reliable image data and accurate interpretation for diagnosis in the clinical laboratory. The standard operating procedure (SOP) for light microscopes includes performance procedure, brief information of all mechanical parts of microscopes with systematic troubleshooting mechanism depending on the laboratory capacity. Maintenance program encompasses cleaning objective, ocular lenses and inner optics; replacement and calibration of light source; XY sample stage management; point spread function (PSF) measurement for confocal laser scanning microscope (CLSM); quality control (QC) program in fluorescent microscopy; and systematic troubleshooting. Laser safety is one of the concern for medical technologists engaged in CLSM laboratory. Laser safety guideline based on the laser classification and risk level, and advisory lab wear for CLSM users are also expatiated in this overview. Since acquired image data presents a wide range of information at the moment of acquisition, well-maintained microscopes with proper microscopic maintenance program are impulsive for its interpretation and diagnosis in the clinical laboratory.

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INTRODUCTION

Proper use of microscopes related to the clinical diagnosis in pathology and laboratory medicine is the second to none issue to medical technologists. For the last two score years, industry of microscope and microscopy has been splendidly developed with a variety of optical

instruments: wide and dark field, phase contrast, fluorescent, confocal laser scanning, super resolution, atomic force and electron microscopes, for multiple application in biomedical sciences [1-3]. Perfectly maintained microscopes by end user help observing specimen clearly and in detail, and that is connected to the reliable report. Local maintenance program on microscopes is, however, barely introduced to medical technologists although educational curriculum encompasses history, mechanical principle and optical physics. The first development of optical microscopes in Europe during

* Corresponding author: Tae Bok Lee

Confocal Core Facility, Center for Medical Innovation, Seoul National University Hospital, 71 Daehak-ro, Jongno-gu, Seoul 03082, Korea

E-mail: snuh21117@gmail.com

* ORCID: <https://orcid.org/0000-0002-8390-6670>

sixteen and seventeen centuries enabled scientists to observe the biological specimen at the micron scale [4]. A mathematical theory and formula ($d=\lambda/2NA$) related to resolution limit of microscope based on the diffraction of wavelength was introduced by Ernst Abbe in 1873 after which a various type of microscopes was invented and currently, microscopic three dimensional image reconstruction was available by image acquisition using CCD camera mounted on the microscope [5]. Simple microscope is composed of five major parts: objective, illuminator, focusing condenser lens, sample stage and binocular; on the other hand, more complicated systems with additional apparatuses such as optical fiber cable, laser as light source, beam splitter, prism, dichromic mirror and photomultiplier (PMT) detector are needed for the advanced microscopes [6-8]. Scheme of optical physics applied to the light microscopes is based on the properties of light: transmission, reflection, refraction, diffraction, absorption and scattering, in order to minimize interference incurred during optical transmission and also optimize signal to noise (S/N) ratio for clear and distinctive images [9]. Confocal microscopy and developed various fluorescent labelling techniques allowed the contemporary researchers to explore the small world of biology and investigate the specimen from whole tissue tile scan imaging to single molecule tracking in cells [10]. Imaging method is fundamental scientific tool intuitively showing molecular interaction simultaneously based on the localization and distribution in cells unlike other indirect protein detection methods [11]. Nevertheless, resolution limit; 250 nm, of light microscope still remains as a hindrance to overcome for molecule level investigation with clearer and further apparent detailed contour [12]. Super resolution microscopy is the quite recently introduced light microscopic technique in order to enhance resolution of imaging system and overcome an imposed diffraction limit by adopting multiple optical, stochastic and software based image-processing techniques: structured illumination microscopy (SIM), stimulated emission depletion (STED) and stochastic optical reconstruction microscopy (STORM) [13]. In this review,

microscopic performance, maintenance program, image quantification analysis and quality assurance assessment by medical technologists as an end user in the clinical laboratory are intensively discussed. To achieve the integral image data which extremely minimizes a possibility of misinterpretation, and prevent the malfunction of the microscopes, a well-established maintenance program and acquiring the microscopic knowledge by medical technologists are fully required. Understanding of microscopic mechanism depicted in this review guides end users how to correctly operate the light microscopes as per the adequate procedure and cope with trouble shooting. Theories of point spread function (PSF) and confocal microscopy expatiated with a detail information in order to help medical technologists to widen the concept of conventional microscopes not only used in the clinical laboratory but research based investigation for development of alterative diagnosis as well. Laser safety is one of the main concern to deal with for preventing the optical hazard faced by medical technologists in the laboratory. In this article, we present an all common overview of maintenance program in addition to guideline for laser safety in light microscopy utilized in the clinical laboratory.

MAIN ISSUE

1. Light microscopes performance

Light microscopy is broadly distinguished as per the light source and detection method; bright field microscopy and fluorescent microscopy. There are advanced imaging techniques which empathize shapes of objectives for better understand of the sample with highlighted contrast and intensified contour of the surface. The Phase contrast technique allows observers to experience the phase shifts which appears when the light passes the thin layer specimen by separating the illuminating light from the specimen-scattered light [14]. Meanwhile, differential interference contrast (DIC) technique is used for enhancement of contrast in unstained and transparent sample by separating a polarized light source using

Wollaston prisms [15, 16]. Microscopes in the laboratory need a written performance procedure for operating light microscope properly, providing high-quality images and reliable optical screening results which are deeply related to the clinical diagnosis [17]. The standard operating procedure (SOP) for microscopes encompasses purpose, scope, responsibility, accountability, operating procedure, cleaning and maintenance [18, 19]. The SOP has to be placed next to the microscope and promptly updated right after any changes made for its stability and safety of users. Improper use of microscope by inexperienced medical technologists is usually followed by misaligned beam path with image distortion and fatal mechanical damages of microscope. Carrying the microscope holding the stage, eyepiece, coarse and fine focus knob may result in misalignment of mechanical stage, malfunction of tension control system and continuous focusing failure. Prior to observing the microbiology sample with special stain technique targeting specific microorganism for its diagnosis, objective lens should be thoroughly cleaned using lens paper with 70% ethanol to remove the unnecessarily attached bacteria with immersion oil leftovers on the objective lens during the previous observation [20, 21]. Wearing glove is advisory during microscopic performance for the sake of laboratory safety and protection of microscopic optics from finger print and skin oil contamination [22]. After using the microscope, it should be covered with the appropriate antistatic dust cover to prevent dust pileup on the surface of the key optics.

2. Maintenance program

In order to acquire an optimal image using the microscopes, first thing to be considered is “cleaning” of outward optics: ocular lens, objective lens and beam path between ocular lens and objective lens from dust and dried liquid dirt such as immersion oil, mounting solution and cover slide sealer. Those extrinsic artifacts not only cause a low quality image with out-of-focus phenomena in the detector mounted microscopes but also distract medical technologists from reading and counting patient sample

and cells accurately. Microscopic maintenance is divided into daily, monthly and quarterly check-up program as per the user manual and service manual provided by manufacturer [23, 24]. Daily and monthly maintenance are managed by local end user and quarterly maintenance is fulfilled by service engineer from manufacturer or microscopist specialized in optical engineering.

1) Cleaning objective, ocular lenses and other optics

Objective lenses are the most crucial part of light microscopes and those are mounted as revolver type under the sample stage in the inverted microscope or located over the stage in upright microscope. Due to the structural location positioning in the vicinity of the sample stage, objective lenses are easily exposed to scratches. To prevent the damage and prolong the lifespan of the microscope, the objective lens turret should be located in level “zero” position in z-axis using the coarse focus knob before changing objective lens to other magnification or after completing the microscopic observation. For cleaning the ocular lenses, use the moisten lens paper with distilled water and gently wipe in a clockwise. On the other hand, it is recommended to avoid cleaning frequently the objective lenses since the objective lenses are qualified with anti-reflection coating made of magnesium fluoride (MgF_2), silicon (SiO_2) or titanium dioxide (TiO_2) for protection of lens and reduce the light loss by reflection [25]. In order to remove smudges or dirt built up on the oil lenses without leaving streaks or residue, isopropyl alcohol diluted with distilled water at 7:3 ratio is advisable. Organic solvent such as acetone, xylene and absolute ethanol are strictly prohibited for cleaning the objective lenses since those solvents possibly dissolve the cement holding the front lens on to the lens barrel and also leave a streak and thin film on the surface of the lens after dried [26, 27]. Dust accumulated in the fluorescent filter cube or bright field condenser filter set can be removed using a puff-duster in a several repeated motion.

2) Light sources

Most of the light microscopes adopts incandescent light

bulb emitting electric light by heating filament with a high temperature (over 250°C on the inside of the glass envelope). The incandescent lamp is divided into several types as per the source of filament which glows with visible light in the glass envelope filled with inert gas which extends protection of filament from oxidation [28, 29]. The statistical life expectancy of microscopic light sources varies depending on the physical thickness of filament and power of electricity: voltage and wattage. For example, filament (3.0×3.0 mm) with 100 watts power and 12 volts in halogen lamp can last for about 600 hours [30, 31]. In order to track the hours of usage of halogen lamp and avoid the low quality image due to the wane filament with flickering light, daily log book with total hour should be kept by end user. The simple method to predict the condition of lamp for its replacement is as follows; (a) flickering, (b) prolonged time for turning on the lamp and (c) slowed reaction of the bulb to increase or decrease of intensity power dial. After exchanging the lamp according to the manufacturer's instruction, alignment should be fulfilled for optimal imaging results. For simple alignment by end user could be achieved as follows; (a) replacing an appropriate lamp matching criteria such as voltage, wattage and pin size; (b) placing white paper on the stage; (c) reducing power intensity of the lamp and if allowed, use red filter cube or eye protection goggles to avoid exposure to UV light; (d) the image of arc or circle on the white paper should be centered by adjustment of reflecting mirror inside of lamp housing using a proper hex key.

3) Sample stage

Sample stage should be properly maintained in the following three ways: (a) cleaning with 70% ethanol after going over the contagious sample in order to prevent cross contamination and for a sake of laboratory safety; (b) dusting off left over dried-up powders of mounting solution and coverslip sealer so that protecting the objective lenses and linear sliding rails of motorized stage from adhering of crumbles; (c) keeping the linear sliding rail of motorized stage greasy by adding lubricant

periodically for accurate and precise movement without frictional heat. Besides, avoid stocking slides or heavy stuffs on the galvanometer XY stage which is used for fine tune of z axis resolution for 3D image acquisition.

4) Point spread function (PSF) measurement

PSF is the 3D diffraction pattern of fluorescent light emitted from a point object in the specimen after excitation and transmitted to the image plane through the objective with specific numerical aperture (NA) [32]. Theoretically, the light emitted from the specimen is collected by the objective lens and focused at the corresponding point in the image plane [33]. Owing to the diffraction of light, the object observed is shown larger and more blurred out than the real size of the molecule. There are two main factors which affect the PSF: wavelength and NA. Shorter wavelength closer to UV (405 nm) showed comparatively smaller PSF compared to longer wavelength (633 nm) [34]. On the other hand, PSF is getting smaller with far better resolution by adopting higher NA objective lenses. The theoretical PSF can be

Figure 1. Diagram of confocal microscope system and conventional fluorescent microscope. Confocal microscope diagram. Cells are excited by laser via objective lens. The only focused section area; focal plane, is imaged using an aperture; pinhole, and photon is detected by photomultiplier detector (PMT). To avoid blurred images and acquiring z-section images for 3D image rendering, confocal laser scanning microscope (CLSM) was used. On the other hand, CMOS camera was used for fast image acquisition during live cell imaging to lessen photo bleaching and laser cytotoxicity on the specimen.

displayed based on the diffraction pattern depending on wavelength and NA. The center of Airy disk with high peak of intensity and surrounding concentric rings with lower intensity reflect best focused center area and blurred outskirt ripples. Higher resolution images can be achieved using higher NA lenses with lower FWHM [35]. In order to calculate the PSF, fluorescent micro bead size between 100~200 nm should be imaged using a PLA APO 100× 1.4 NA lens with the 1 Airy unit pinhole setting in the confocal laser scanning microscope (CLSM) [36]. The choice between CLSM and conventional fluorescent microscope for investigation of fluorescence label in the sample is made based on the purpose of the experiment. The CLSM gives clearer and more detail images with fine focal planes compared to the fluorescent microscope by adopting sophisticated optical aperture; pinhole and excitation specific light source; laser. On the other hand, con-

ventional fluorescent microscope images blurred wide field phase which includes not only focal plane phase but out-of-focus z-axis region (Figure 1). The point like image of micro bead is shown larger than real size because of the diffraction of light. In 2D image of XY axis, center spot and concentric diffraction rings are observed; on the other hand, much elongated oval shape of diffraction pattern out of center spot is shown in Z axis.

*Airy disk: central bright circular region with surrounded concentric rings manifested in the best focused spot of light.

*FWHM: Full width at half maximum (FWHM) is the distance between two curve points at the half maximum level from the peak.

*Rayleigh limit: a criterion where two minimum resolvable details are distinguished as independent objects.

Table 1. Microscopic errors and proper troubleshooting

Problems	Troubleshooting
No light	Beam splitter check (Pull out position) Power supply check Light intensity controller check Iris diaphragm check Objective turret position check Binocular safety shutter check Lamp filament check Fluorescent filter cube check
Dark images	Increase light intensity Open iris diaphragm Select appropriate phase contrast filter as per the objective lens Condenser adjustment Camera or detector gain and exposure time adjustment
Too bright images	Decrease light intensity Close iris diaphragm and condenser adjustment Select appropriate phase contrast filter as per the objective lens Reduce detector gain or camera exposure time
Focusing error	Common check: Check coarse knob (adjusting focusing tension gear: clockwise to tighten; counter-clockwise to loosen tension) Coverslip should face the objective lens (inverted microscope: slide glass upside down, upright microscope: slide glass upright position) Lens cleaning (objective and binocular) Water or oil lenses: One drop of water or immersion oil on the lens for prevention of refraction of light
Artifact stuck in the field of view	Clean eyepiece, objective and condenser optics Disassemble eyepiece and bluff out dust Disassemble lamp housing and bluff out dust
Image distortion	Lopsided image: Sample stage calibration Dark and blurred image at outer surrounding: Adjust iris diaphragm

5) Quality control program in fluorescent microscopy

QC program in microscopy is somewhat difficult to establish due to lack of adequate and stable QC sample for imaging. For the fluorescent microscopy, especially, QC sample should be freshly prepared almost every day for its assessment and the stain quality of QC slides prepared by end user are easily affected by skills and confluence of technologists. A commercially available prepared microscope slide; convallaria, lily of the valley of rhizome with concentric vascular bundles, demonstrates autofluorescence and that is detected from the vicinity of ultra violet; 405 nm to deep red; 633 nm without dramatic attenuating of its fluorescence under consecutive excitation by light sources. The stability of light source, linearity of beam path and optimized functionality of detecting mechanism are in most of the times attained by manufacturer provided service engineers. However, those essential check-up for maintaining the finest state of microscopes in the laboratory can be indirectly achievable using a convallaria sample slide. Daily acquired images for QC program can be evaluated and assessed in an intensity based manner, using ImageJ, a Java-based image processing program, developed by National Institute of Health (NIH) and Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin) [37].

6) Troubleshooting

A variety of mechanical and system errors occur during

microscopic operation: placing slide glasses, observing samples, adjusting focus and sample stage, capturing images and carrying microscopes. To prevent critical disorder and reduce preventable miscellaneous mistakes, an appropriate education on troubleshooting in microscopy is required. Comprehensive understanding on the light microscopic components with an accurate solution to the problem is related to efficient handling of the instrument for investigative and diagnostic purposes [38]. The systematic approach to solve common mechanical predicaments emerged during microscopic operation are expatiated for end users in the clinical laboratory (Table 1).

3. Laser safety

Direct exposure of naked eyes to the light source causes irreversible damages of eyesight. Thermal effects and photo-chemical effects caused by laser exposure to eyeball are the major issues in laser radiation and safety. Most of the confocal microscopes which adopt laser as a light source in lieu of white light, is potentially hazardous when users directly look at the laser beam passing through the objective lens. Radiation with UV laser around 400 nm damages cornea and causes cataracts. On the other hand, consecutive direct exposure to the infrared laser over 700 nm which can penetrate the lens of eyeball increases a possibility of irreversible damages in retina [39, 40]. The laboratory and safety manager should assess the laser safety as per the international standard assuming the

Table 2. Laser classification and risk level

Classification	Hazardous	Application	Risk level
Class 1 (220~400 μ W)	Not hazardous Safe during normal operation	Data reading system in CD/DVD, laptop, PC	Low
Class 2 (1 mW)	Safe due to the blink reflex (glare aversion response to bright lights) Visible light lasers only (400~700 nm)	Presentation laser pointer, Barcode reading system in clinical chemistry and hematology equipment	Low-Medium
Class 3R (5 mW)	Unsafe with low risk of eye injury Avoid direct exposure to eyes.	Measuring and targeting devices, Intensified power pointer	Medium-High
Class 3B (500 mW)	Unsafe for eyes (Direct viewing can cause eye injury, diffuse reflections are not hazardous) Generally safe for skin	Confocal microscopes, Flow cytometry	High
Class 4	Unsafe for eyes Unsafe for skin	STED (super resolution microscope), Medical lasers, Industrial cutting/welding devices, Laser show projectors	Extreme

worst-case scenario of exposure of laser with maximum intensity power (Table 2). The Lasershield (180~400 nm OD 5+, 400~700 nm OD 1), laser safety goggles, manufactured by NoIR (www.noirlaser.com) is also recommended when the users maintain the light source for its calibration.

CONCLUSION

Light microscopes used in the clinical laboratory are surely in the middle of controversial issues with respect to the quality control and management thereof. To present the feasible and practical approaches in the microscopic management and simple QC program performed by medical technologists in the clinical laboratory, the various examples from basic management techniques to theoretical optical physics mechanism in addition to laser safety should be dealt with for the comprehensive understanding of end-user. Because of the effects of diffraction, a misaligned microscope is the main cause of distorted and false images which may lead to the misinterpretation of patient slide samples and wrong diagnosis. Moreover, a misaligned and malfunctioning microscope is deeply related to the poor working environment for medical technologists during the observation. Properly aligned and adjusted optical compartments of the microscope: specimen stage, focus, objective, eyepieces, lamp, filter cube and related inner optics, are essential for the accurate image data analysis followed by clinical diagnosis [41]. The best image from the clinical sample is deeply related to the reliable decision and diagnosis and that always comes from well-maintained microscopes and well-trained eyes of medical technologist with skillful microscopy. QC program for the light source and detector in fluorescent microscope is hard to establish owing to lack of proper and stable QC sample for daily image acquisition. However, a commercially available convallaria slide exhibits stable autofluorescence and that is useful to set up the instrument QC program for simple monitoring a light source power and detector sensitivity [42]. Medical technologists engaged in the CLSM laboratory should be aware of the laser safety

guideline and knowledgeable about wavelengths of power levels in each laser equipped in the microscope [43]. In this review, we discussed a variety of microscopic issues related to performance, maintenance and laboratory safety which are mainly involved in the clinical laboratory science for the diagnosis of diseases by naked eyes of medical technologists. A plenty of information on microscopy and its maintenance program varies from simple technical tips to advanced optical and mechanical theories. For more practical application and efficient performance with microscopes in local clinical laboratories, fundamental technical reports and books were severely screened, selected and expatiated. Widely used light microscopes in clinical laboratories are under responsibility of medical technologists and qualified image data are related to the laboratory credentials. Well-established microscope and microscopy SOP and maintenance program with related laboratory safety guidelines are necessary in order to acquire a linearity of image data between control and test images; achieve analytical and statistical correlation of image data which always come from well-maintained microscope and intuition of trained medical technologists; and prevent unnecessary irreversible visual impairment of observers.

요약

임상병리 검사분야에 있어서 환자로부터 유래된 조직이나 세포의 형태학적 변화, 세포 생리, 세포 내 분자의 추적 및 신호 전달 체계 등의 임상검사 및 관련 연구를 위한 빼놓을 수 없는 중요한 진단과 연구장비로서 현미경이 가지는 의미는 크다고 할 수 있다. 현미경에 대한 포괄적인 지식과 이해를 바탕으로 현미경의 올바른 사용, 관리와 유지보수는 신뢰도 높은 이미지 획득과 그에 따른 정확한 데이터 분석을 통한 질병의 진단을 위해서 반드시 요구되는 부분이라고 할 수 있다. 광학현미경의 표준 운영 절차(standard operating procedure, SOP)는 현미경의 작동 절차와 함께 검사실 규모에 따른 현장 사용자의 체계적인 현미경 장애 해결 방안과 기계적 원리에 대한 핵심 정보가 함께 수록되어야 한다. 현미경 유지관리 업무에는 대물, 접안렌즈와 현미경 내부 광학필터의 청소, 광원의 교체와 교정, XY재물대 유지보수, 공초점 레이저 주사 현미경(confocal laser scanning

microscope)에서의 점확산함수(point spread function, PSF) 측정, 형광현미경에서의 검사 품질관리(quality control, QC)와 체계적인 현미경 장애 해결방안 등이 포함되어야 한다. 본 중설에서는 국제적 기준에 따른 레이저의 위험도에 따라 일부 현미경에 장착된 레이저 광원에 대한 안전지침과 보호장구에 대한 내용을 함께 소개하였다. 현미경을 통해 획득된 이미지는 촬영된 시점의 검체에 대한 모든 정보를 제공한다고 할 수 있으며, 적절한 유지보수 프로그램과 그에 따라 적합하게 관리된 현미경만이 이미지 데이터를 통한 정보의 획득, 올바른 해석과 정확한 진단에 반드시 필요한 선제 조건들이라고 하겠다.

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