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Standard Guide for Visualization and Identification of Nanomaterials in Biological and Nonbiological Matrices Using Darkfield Microscopy/Hyperspectral Imaging (DFM/HSI) Analysis[1]

This standard is issued under the fixed designation E3275; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide has been prepared to familiarize laboratory scientists with the background information and technical content necessary to image and identify engineered nanomaterials (ENMs) in cells via darkfield microscopy/hyperspectral imaging (DFM/HSI) methodology.

1.2 DFM/HSI is a hyphenated bioanalytical technique/tool that combines optical microscopy with high-resolution spectral imaging to both spatially localize the distribution of and identify ENMs within a suitably prepared test sample.

1.2.1 In the context of mammalian cells, ENMs will have distinctive light-scattering properties in comparison to subcellular organelles and cell structural features, which can allow one to discriminate between the spectral profiles of ENMs and cellular components.

1.2.2 The light-scattering properties of ENMs in other test samples, such as fixed tissues, plants, complex drug product formulations, filter media, and so forth, will also be different from the native matrix component scattering signals inherent to these other types of samples, thus allowing for ENM visualization and identification.

1.3 This guide is applicable to the use of DFM/HSI for identifying ENMs in the matrices mentioned.

1.4 This guide describes and discusses basic practices for setting up and using DFM/HSI instrumentation, sample imaging techniques, considerations for optics, image analysis, and the use of reference spectral libraries (RSLs). DFM/HSI is routinely used in industry, academia, and government as a research and development and quality control tool in diverse areas of nanotechnology.

1.5 The values stated in SI units are to be regarded as the standard. No other units of measurement are included in this standard.

1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

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2. Referenced Documents

2.1 *ASTM Standards:*[2]

E2456 Terminology Relating to Nanotechnology

E3255 Practice for Quality Assurance of Forensic Science Service Providers Performing Forensic Chemical Analysis

2.2 *ISO Standards:*[3]

ISO 20473 Optics and photonics—Spectral bands

ISO/TS 80004-1 Nanotechnologies—Vocabulary—Part 1: Core terms

3. Terminology

3.1 *Definitions:*

3.1.1 *agglomerate, n*—group of particles held together by relatively weak forces (for example, van der Waals or capillary) that may break apart into smaller particles upon processing. **E2456**

3.1.2 *aggregate, n*—discrete group of particles in which the various individual components are not easily broken apart, such as the case of primary particles that are strongly bonded together (for example, fused, sintered, or metallically bonded particles). **E2456**

[1] This guide is under the jurisdiction of ASTM Committee E56 on Nanotechnology and is the direct responsibility of Subcommittee E56.08 on Nano-Enabled Medical Products.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, http://www.ansi.org.

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3.1.3 *blank sample, n*—sample matrix carried through all or part of the analytical process, where the analyte is not present, or where the analyte response is suppressed.

3.1.4 *blocking negative ligand control sample, n*—control sample that is treated with free ligand at an appropriate concentration to reduce the number of available binding sites for the targeted ENM.

3.1.5 *camera gain, n*—conversion ratio between the detected electrons (e^-) and the digital number of the digitized signal output by the camera.

3.1.5.1 *Discussion*—For example, a gain of 4 means that it takes 4 e^- to produce one analog-to-digital unit (one "count") in the camera output.

3.1.6 *dark current, n*—rate of increase of unwanted electron signal that get added to a pixel's signal, usually expressed in e^- per second per pixel.

3.1.6.1 *Discussion*—Longer acquisition times result in larger dark signal and more contribution to noise. Higher temperature also increases the dark current, which is why most scientific cameras are cooled (sometimes down to -100°C) to minimize dark current and the associated contribution to total noise.

3.1.7 *darkfield condenser, n*—device that provides sample illumination that is only detected by the detection objective in the presence of a scattering object in the sample and is usually achieved by oblique illumination of the sample.

3.1.8 *darkfield microscopy, DFM, n*—high-contrast light microscopy technique that excludes unscattered light in which scattering bodies appear bright against a black background.

3.1.9 *data cube, n*—array that consists of data, which includes spatial (*x*, *y*) and spectral information.

3.1.10 *engineered nanomaterial, ENM, n*—nanomaterial designed for a specific purpose or function. **ISO/TS 80004-1**

3.1.11 *field of view, FOV, n*—spatial view of the sample that is obtained after acquisition.

3.1.11.1 *Discussion*—The FOV is instrument dependent.

3.1.12 *full well, n*—maximum number of electrons that can be held in a single pixel of a camera sensor.

3.1.12.1 *Discussion*—If more photons strike a full pixel during an exposure, the signal output remains the same and the pixel is said to be saturated.

3.1.13 *hyperspectral imaging, HSI, n*—imaging technique in which the full absorption, emission, or reflection of light is collected for each spectral band or wavelength.

3.1.14 *hyperspectral mapping, n*—classification of points within a field of view using similarities between the spectra within the reference spectral library and with the spectrum of the unknown material.

3.1.15 *hyperspectral system, n*—system consisting of appropriate light sources and detectors that can measure a range of wavelengths typically including visible near infrared (VNIR), short wave infrared (SWIR), and sometimes near ultraviolet (UV).

3.1.16 *image cube, n*—hyperspectral image composed of concatenated two-dimensional spatial images at a single spectral frequency.

3.1.17 *nanomaterial, n*—material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale. **ISO/TS 80004-1**

3.1.18 *nanoparticle, n*—classification of an ultrafine particle with lengths in two or three dimensions greater than 1 nm and smaller than approximately 100 nm and which may or may not exhibit a size-related intensive property. **E2456**

3.1.19 *near ultraviolet, near UV, n*—electromagnetic spectrum containing the wavelength range 315 nm to 380 nm. **ISO 20473:2007**

3.1.20 *negative control, n*—a material of established origin that is used to confirm that a procedure does not produce an unintended result. **E3255**

3.1.21 *quantum efficiency, QE, n*—ratio of the number of photons detected as signal to the total number of photons striking the camera sensor.

3.1.21.1 *Discussion*—Photons detected as signal are the ones that get converted to electrons and then read out as signal by the sensor electronics. The higher the QE of the camera sensor, the shorter the measurement time for the same total number of detected photons and therefore the higher the signal to noise per unit time. QE is wavelength-dependent, so it is important to verify that the camera sensor has sufficiently high QE over all wavelengths of interest.

3.1.22 *read noise, n*—error that gets added to each pixel signal upon read out of a camera sensor, usually given in terms of electrons (e^-).

3.1.22.1 *Discussion*—The lower the readout noise, the less it affects the quality of the signal.

3.1.23 *reference spectral library, RSL, n*—collection of the full absorption, emission, or reflection of light from known materials combined into a spectral library used to identify and map unknown materials within a hyperspectral image.

3.1.24 *short wave infrared, SWIR, n*—electromagnetic spectrum containing the wavelength range 1400 nm to 3000 nm. **ISO 20473:2007**

3.1.25 *shot noise, n*—statistical error caused by the discrete nature of the photons striking a sensor.

3.1.25.1 *Discussion*—This noise is inherent to the physical nature of light and cannot be reduced by improving the quality of the camera electronics.

3.1.26 *spectral library, n*—collection of the full absorption, emission, or reflection of light of known materials, including those within specific matrices, collected by a hyperspectral system that may show matrix-dependent spectral features.

3.1.27 *spectrum cube, n*—a 3-dimensional array of simultaneously acquired 2-D images with spectral information for each image contained in the third dimension; a spectrum cube consists of a series of independent acquisitions, ranging from 1 to n where n is defined as an unlimited field of view, in which all spatially resolved spectral information is obtained simultaneously.

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3.1.27.1 *Discussion*—The third dimension is the distance of the segment along the field of view. The final hyperspectral image is dependent upon the data processing software utilized.

3.1.28 *targeted ENMs, n*—nanomaterials that have a targeting ligand attached on the outer surface.

3.1.29 *treated sample, n*—sample that has been exposed to one or more types of ENMs.

3.1.30 *treated negative ligand control sample, n*—a cell-based sample which does not contain a targeting ligand; this control sample has been exposed to one or more types of ENMs.

3.1.31 *visible near infrared, VNIR, n*—electromagnetic spectrum containing the wavelength range 380 nm to 1400 nm.

ISO 20473:2007

4. Summary of Guide

4.1 A test sample containing ENMs is placed onto a glass microscope slide and protected with a coverslip. In addition, samples that have not been exposed to ENMs (negative controls) and samples that have been exposed to known types and concentrations of ENMs (positive controls) are prepared on corresponding microscope slides. To detect, identify, and determine the spatial distribution of ENMs in the test sample, the sample is illuminated at an oblique angle using a darkfield microscope. Scattered light from the ENMs in the sample is captured in the form of high-contrast microscopy images that enable precise localization of the ENMs. Next, an HSI system is used to obtain and record spectral data for each pixel in the DFM sample images within a specified range, commonly within the VNIR or SWIR ranges. In this manner, spectral libraries are prepared for each test sample. Appropriate control measurements, light corrections, and system calibration procedures to reduce the incidence and impact of matrix artifacts shall be performed with the negative and positive control samples to build a reference spectral library (RSL) (**1, 2**)[4]. The RSL is used in a mapping process to positively identify and determine the spatial distribution of the ENMs in a test sample.

5. Significance and Use

5.1 The information and recommendations in this guide are relevant for imaging and identifying ENMs in cells and other biological (for example, fixed tissues, whole plants) and nonbiological (for example, drug formulations, filter media, soil, and wastewater) matrices after appropriate sample preparation procedures have been performed (**3-5**). DFM/HSI is a recently developed analytical tool; however, the relative simplicity of sample preparation combined with the potential to acquire high-contrast ENM images and high-content ENM spectral responses facilitates the increasing use of the tool for diverse applications in drug delivery, toxicology, environmental science, biology, and medicine.

5.2 Verification of the uptake and spatial distribution of ENMs in cells, for example, is necessary for evaluating and understanding the biological effects of ENMs on living systems. Similarly, the closeness of the spatial distribution of ENMs in complex drug formulations can be an important criterion in establishing physicochemical similarity between formulations (**6**). Complex products are described in the most recent version of the Generic Drug User Fee Act (GDUFA) reauthorization commitment letter: (**7**). This guide covers the criteria and general considerations for performing DFM/HSI analyses on samples of biological and nonbiological origins containing ENMs (for example, metal and metal oxide nanoparticles, or carbon nanotubes, or both). This guide does not cover or address provisions for imaging or identifying, or both, non-engineered (natural) nanoparticles/nanomaterials in cells or other matrices, nor does this guide describe or discuss the application of DFM/HSI for determining the dimensions of ENMs.

6. Instrumentation

6.1 *General Instrument Description:*

6.1.1 A DFM system is composed of an optical imaging microscope with a series of direct light blocks and high angle mirrors which only allow for light scattered at high angles to be detected. The imaged areas where the sample is absent appear dark; hence, the name darkfield imaging. A DFM/HSI system detects the scattering spectrum of the sample at each spatial point of the image.

6.1.2 Sample illumination is typically performed using a broad-spectrum light source, and the light scattered by the sample is then separated by wavelengths before reaching the image sensor.

6.2 *Microscope Type:*

6.2.1 DFM is typically performed on upright or inverted microscopes that differ from each other in the orientation of the illumination, sample, and objective.

6.2.2 In the case of upright microscopes, the transmitted illumination is directed upwards from underneath the sample and the microscope objective is located above the sample. Image focus is typically performed by moving the sample stage vertically with the objective lenses in a fixed position while focusing.

6.2.3 In the case of inverted microscopes, the transmitted illumination is located above the sample and pointed downwards. The objective is located under the sample. The height of the sample stage is fixed and focusing is performed by moving the objective vertically.

6.3 *Light Sources:*

6.3.1 A typical light source for DFM/HSI shall be bright and cover the wavelength range of interest. Ideally, the spectrum of light emitted from the source should be flat and devoid of sharp spectral features to facilitate subsequent data processing. Typical examples of light sources for darkfield imaging are given below.

6.3.1.1 Halogen lamps, because of their low cost, are standard lighting sources found on most microscopes. The typical spectrum from a halogen lamp (Fig. 1) is devoid of peaks and approximately follows the spectrum of a blackbody radiator of equivalent temperature.

6.3.1.2 Light-emitting diode (LED) lamps are increasingly popular for their electrical efficiency and small amount of

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[4] The boldface numbers in parentheses refer to a list of references at the end of this standard.

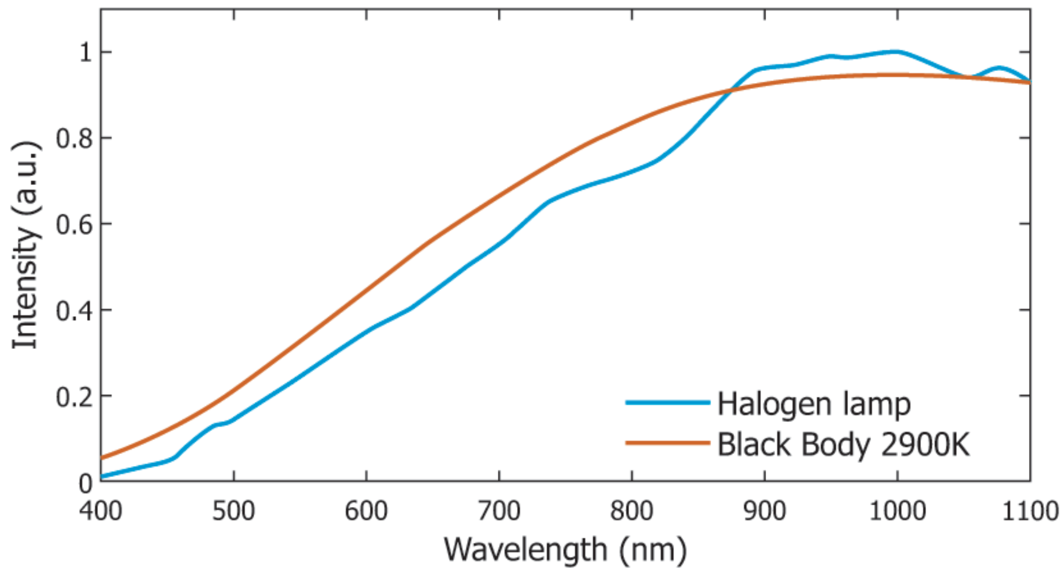

FIG. 1 Example Spectrum from a Tungsten Halogen Lamp

generated heat. Typically, white LEDs have a spectrum characterized by a peak around 450 nm and a broad peak centered approximately at 550 nm (Fig. 2). The light output around 400 nm and above 700 nm is generally poor. The different peaks in the spectrum of LED sources make HSI more difficult as the peaks must be taken into account during analysis of the acquired hyperspectral data.

6.3.1.3 Xenon arc lamps are characterized by a relatively smooth emission up until 800 nm (Fig. 3). Above that wavelength, large peaks appear that complicate analysis of the hyperspectral images.

NOTE 1—These three different light sources can all be used for DFM/HSI; however, xenon light sources provide better resolution for ENMs due to the small peaks and valleys in the short wavelength range (400 nm to 800 nm).

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6.4 *Microscope Condenser*—The condenser is the optical element that collects the light emitted from the source and redirects it to the sample. A darkfield condenser ensures that the light reaches the sample at an oblique angle leaving an “empty cone” of light along the detection axis of the microscope objective. This empty cone of illumination sets the limit of the numerical aperture (NA) of the microscope objective that can be used for detection. There are two categories of darkfield condensers: dry and oil immersion, which are described in 6.4.1 and 6.4.2.

6.4.1 The dry darkfield condenser is used without oil immersion and is located a few millimetres from the sample. It typically illuminates the sample with a NA from ~0.8 to 0.95, which means that any objective with a NA below 0.8 should be

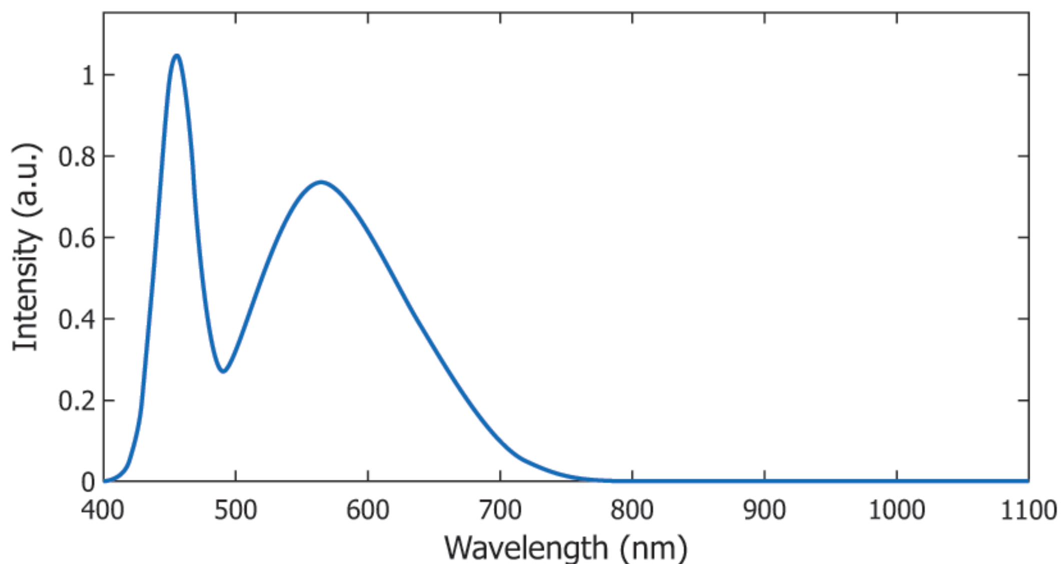

FIG. 2 Example Spectrum from an LED Lamp

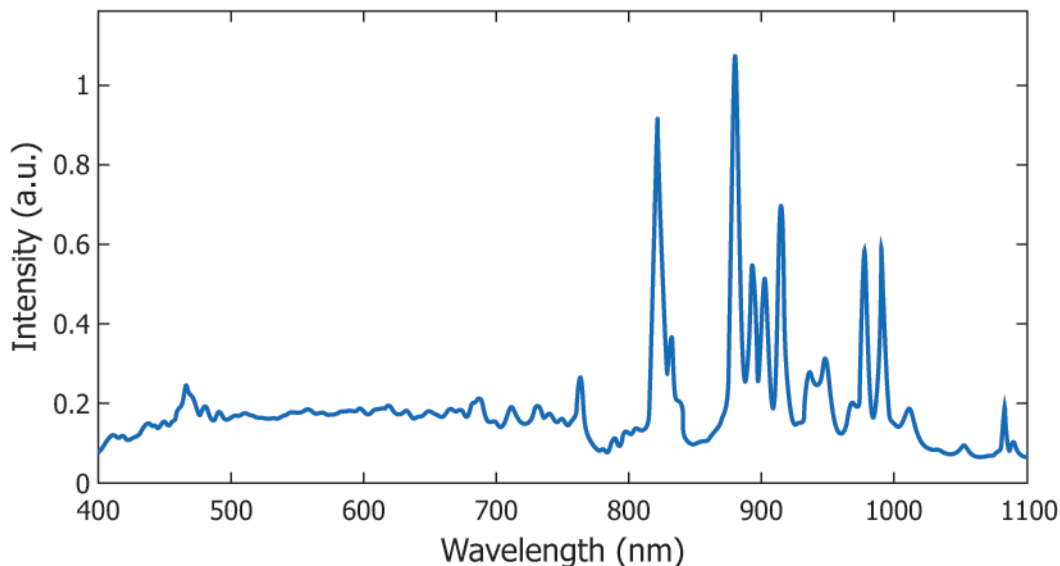

FIG. 3 Example Spectrum from a Xenon Arc Lamp

suitable for darkfield imaging (in practice, NAs ~0.7 and lower are recommended to avoid parasitic light caused by potential system misalignment).

6.4.2 The oil immersion darkfield condenser illuminates the sample with a much larger NA, typically above 1.2, which permits the use of objectives with a larger NA than allowable with the dry condenser. However, this condenser uses immersion oil between the sample and the condenser, which can affect some samples and requires frequent cleaning.

6.5 *Microscope Objectives*—Microscope objectives are a central part of the instrument. The components of two types of objectives are shown in Fig. 4. The user should understand and review all the characteristics of the objective to ensure optimal performance of the instrument.

6.5.1 Magnification is the size of the sample's image in the instrument image plane relative to the real size of the sample. The objective magnification fixes the FOV of the instrument and the corresponding size of the imaging pixels on the sample. In general, the user chooses the magnification as a compromise between the desired size of the FOV and the desired spatial resolution, that is, the larger the FOV, the poorer the spatial resolution.

6.5.2 The numerical aperture indicates the resolving power of the objective and also its light-gathering capacity. Larger NA objectives (~0.5 to 1.2) provide better spatial resolution and better light collection efficiency. However, larger NA objectives result in shallower depths of field, which may be undesirable for non-flat samples such as cells. Care shall also be taken to ensure that the NA of the objective is lower than the limit set by the darkfield condenser that is used.

6.5.3 Working distance (WD) is the distance between the front of the objective and the sample. The user should choose an objective with a sufficiently large WD to account for the thickness of the coverslip or the microscope slide thickness, depending on the sample orientation.

6.5.4 Correcting ring is a feature found on some objectives that is used to vary its aberration correction to account for different coverslip thicknesses.

6.5.5 Iris ring is a feature found on some objectives that is used to vary the NA value to control the spatial resolution, depth of field, and light collection efficiency. In DFM, this feature is useful to reduce the NA of the objective to a value lower than the limit set by the condenser.

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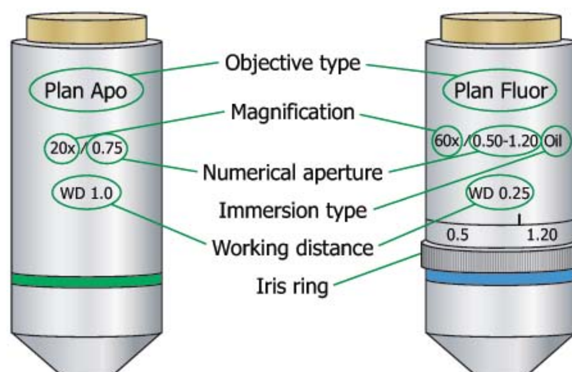

FIG. 4 Important Physical Characteristics of a Microscope Objective

6.5.6 Immersion media are required by some objectives to work correctly. The immersion media used will depend upon the analyzed samples and objectives. For ideal illumination, the immersion media should be matched with the refractive index of the condenser and sample matrix.

6.5.7 *Objective Type*—Microscope objectives are designed to correct for different optical aberrations to increase image quality. Plan achromatic and apochromatic (APO) objectives correct for chromatic and field curvature aberrations with APO objectives providing better chromatic aberration correction. These corrections are crucial for HSI of ENMs in which two ENMs that may scatter at different wavelengths shall both be in focus at the same time within the field of view.

6.6 *Widefield Tunable Filter*—A widefield tunable filter is a bandpass-imaging filter for which the central wavelength can be selected by the user. For HSI, a sequence of images is acquired at different wavelengths to construct an image cube. See Section 7 for more details. Important specifications of the tunable filter are its NA, spectral width of the bandpass filter, and the range of wavelength accordability.

6.7 *Imaging Spectrometer*—An imaging spectrometer images a line on the sample and disperses the wavelengths on the axis perpendicular to the line on a two-dimensional (2D) sensor. For HSI, a sequence of lines is acquired at different positions on the sample to construct a spectrum cube; see Section 7 for more details. Important specifications of the imaging spectrometer are its spectral resolution and its f-number (F#, which is related to its light-gathering capacity).

6.8 *Camera*—HSI typically uses monochromatic cameras. For ENM imaging, the visible and NIR wavelengths are of interest, with silicon-based image sensors covering the 200 nm to 1100 nm wavelength range. Although less often encountered, the 900 nm to 1700 nm range can be probed with indium gallium arsenide (InGaAs) cameras. In all cases, there are many specifications for cameras that the user should understand to ensure optimal camera choice and performance metrics such as quantum efficiency, gain, full-well capacity, read noise, and dark current. See Section 3 for more details on these metrics.

NOTE 2—It is not practical to operate a commercial microscope at wavelengths less than 360 nm.

7. Image Capturing/Optics

7.1 *Optical Methodologies*—HSI systems used in ENM characterization are presently available in two formats.

7.1.1 *Wavelength Dispersive Models:*

7.1.1.1 Instruments based on wavelength dispersive models use a diffraction grating or prism to disperse the spectrum and are also referred to as "line-scan" or "push-broom" systems. The FOV is imaged onto the entrance slit of a wavelength dispersive spectrometer (WDS) that uses a 2D detector such as a complementary metal-oxide semiconductor (CMOS) or charge-coupled device (CCD) camera. The only light that passes through the WDS is the slice of the FOV that passes through the entrance slit of the WDS (see Fig. 5). Spectra are dispersed along rows of pixels and spatial information along columns of pixels. Each acquisition is an independent 2D mapping and contains spatially resolved spectra of each point along the entrance slit. These "points" along the entrance slit correspond to "points" along the segment of the FOV. Each acquisition can be thought of as a "spectral snapshot."

7.1.1.2 The sample is moved perpendicular to the entrance slit by a translation stage under computer control. This action is arbitrarily repeated sequentially as often as required to complete a user-defined FOV. The spatial *y*-axis is determined by the height of the entrance slit. To create a hyperspectral image, independent spectral snapshots are concatenated together to form a "spectrum cube;" see Fig. 6. An image cube is

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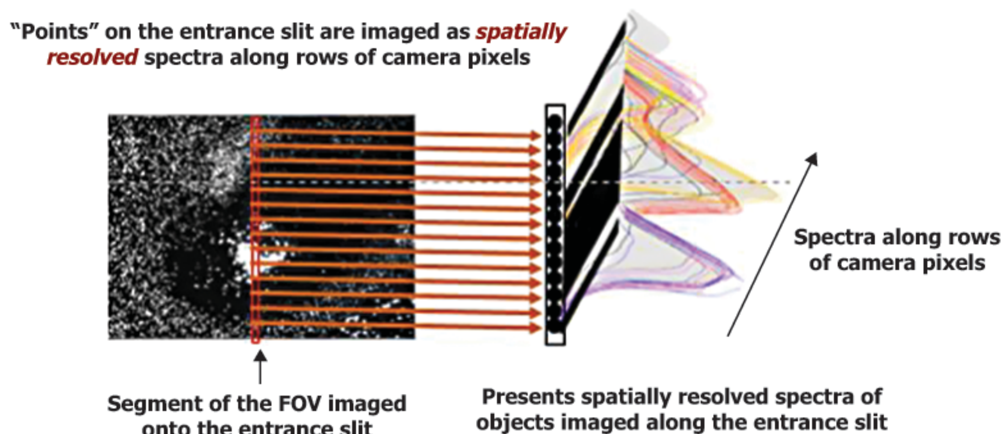
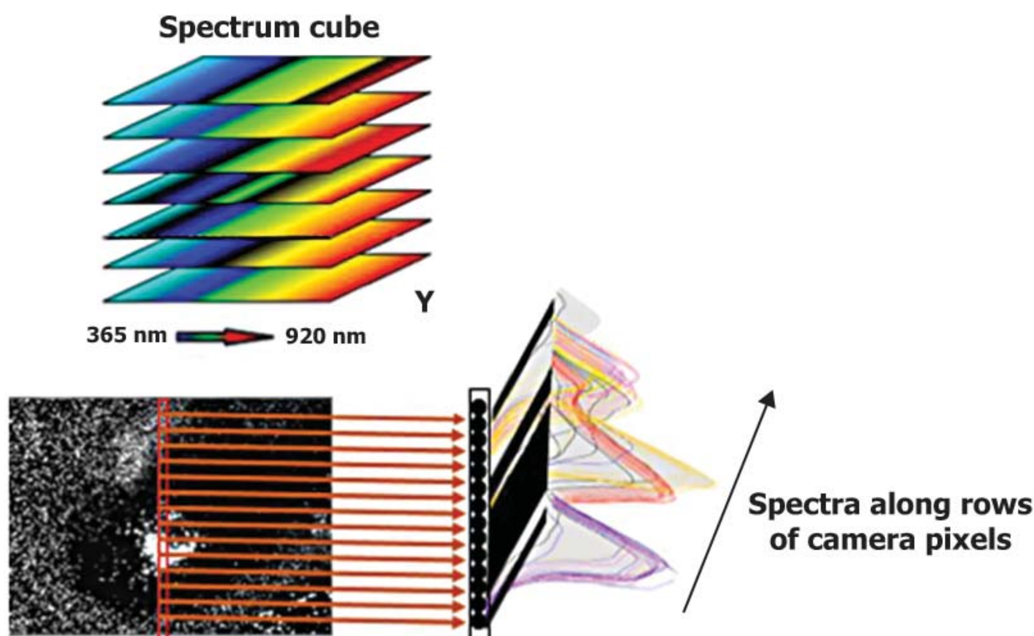


FIG. 5 Example of Wavelength Dispersive HSI System Used for Interrogating the Presence of ENMs in Biological and Nonbiological Matrices



Each "slice" of the cube presents spatial resolution along the "Y" axis and spectra along rows of camera pixels the "X" axis.

Each slice is independent with respect to spectral information. Sequential spectral acquisitions join to render an unlimited FOV.

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FIG. 6 Example of Wavelength Dispersive HSI System Used for Interrogating the Presence of ENMs in Biological and Nonbiological Matrices

created when a fixed FOV is measured at a series of independent sequential wavelengths and shall be completed to acquire full spectral content. A single acquisition or element in the image cube produces a "picture" of the entire FOV at just one wavelength. Conversely, a spectrum cube is generated by WDS systems, and each single acquisition presents a spatially resolved slice of a FOV at all wavelengths simultaneously. A slice or "segment of the FOV" is defined as the height and width of the entrance slit divided by the magnification of the microscope objective. Spatially resolved spectra produced along the slit are captured along rows of pixels in the camera consistent with the image of the height of the entrance slit. Various file formats can be used specific to either data cube format including, but not limited to, tagged image format file (TIF) with extended metadata, file information tool set (FITS), and hierarchical data format (HDF) HDF4, and HDF5. All of these formats can be read by various image analysis software for further processing. Software to convert spatially resolved spectral data into an image can be specific to the instrument developer or procured by commercial packages.

7.1.2 *Tunable Imaging Filter Models*—Instruments based on tunable image filter models use acousto-optical tunable filters (AOTF), liquid-crystal tunable filters (LCTF), interferometers, or hologram-based wavelength tunable devices to produce a filtered image of the FOV captured by a 2D image sensor and are often referred to as "staring" devices. In this model, sequential acquisitions are made of the same FOV at different wavelengths through a tunable imaging bandpass filter. A scan of 100 acquisitions will result in 100 images of the FOV at each wavelength. Each pixel of the camera measures a spectrum of one point in the FOV one wavelength after another. Once completed, the output from this process is termed an "image cube;" see Fig. 7. No translation of the FOV is necessary in the case of these "staring" devices. Movements of the FOV or objects within it can be compensated by post-processing registration algorithms. Software to convert spatially resolved spectral data into an image can be specific to the instrument developer or obtained by commercial "off-the-shelf" packages. There are also open-source tools built on a variety of platforms such as Python, Octave, and R that may be customized to specific needs of advanced users.

8. Sample Imaging

8.1 *General Considerations*—For every instrument, the user should set up the microscope for darkfield observation of the sample before hyperspectral measurements can be started. The microscope setup differs slightly depending on whether the microscope is upright or inverted, and whether the condenser, objective or both are dry or oil immersion. Generally, an upright microscope is preferable for darkfield illumination. Not