



Designation: E3275 – 21

Standard Guide for Visualization and Identification of Nanomaterials in Biological and Nonbiological Matrices Using Darkfield Microscopy/Hyperspectral Imaging (DFM/HSI) Analysis¹

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.*

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

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.

Current edition approved June 1, 2021. Published July 2021. DOI: 10.1520/E3275-21.

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>.

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.