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Standard Guide for Performing Quantitative Fluorescence Intensity Measurements in Cell-based Assays with Widefield Epifluorescence Microscopy[1]

This standard is issued under the fixed designation F3294; 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 guidance document has been developed to facilitate the collection of microscopy images with an epifluorescence microscope that allow quantitative fluorescence measurements to be extracted from the images. The document is tailored to cell biologists that often use fluorescent staining techniques to visualize components of a cell-based experimental system. Quantitative comparison of the intensity data available in these images is only possible if the images are quantitative based on sound experimental design and appropriate operation of the digital array detector, such as a charge coupled device (CCD) or a scientific complementary metal oxide semiconductor (sCMOS) or similar camera. Issues involving the array detector and controller software settings including collection of dark count images to estimate the offset, flat-field correction, background correction, benchmarking of the excitation lamp and the fluorescent collection optics are considered.

1.2 This document is developed around epifluorescence microscopy, but it is likely that many of the issues discussed here are applicable to quantitative imaging in other fluorescence microscopy systems such as fluorescence confocal microscopy. This guide is developed around single-color fluorescence microscopy imaging or multi-color imaging where the measured fluorescence is spectrally well separated.

1.3 Fluorescence intensity is a relative measurement and does not in itself have an associated SI unit. This document does discuss metrology issues related to relative measurements and experimental designs that may be required to ensure quantitative fluorescence measurements are comparable after changing microscope, sample, and lamp configurations.

1.4 *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.*

1.5 *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]

E131 Terminology Relating to Molecular Spectroscopy

E284 Terminology of Appearance

E2186 Guide for Determining DNA Single-Strand Damage in Eukaryotic Cells Using the Comet Assay

E2642 Terminology for Scientific Charge-Coupled Device (CCD) Detectors

E2719 Guide for Fluorescence—Instrument Calibration and Qualification

E2825 Guide for Forensic Digital Image Processing

F2944 Test Method for Automated Colony Forming Unit (CFU) Assays—Image Acquisition and Analysis Method for Enumerating and Characterizing Cells and Colonies in Culture

F2997 Practice for Quantification of Calcium Deposits in Osteogenic Culture of Progenitor Cells Using Fluorescent Image Analysis

F2998 Guide for Using Fluorescence Microscopy to Quantify the Spread Area of Fixed Cells

2.2 *ISO Standards:*[3]

ISO 13653 Measurement of relative irradiance in the image field

ISO/IEC 10918-1:1994 Digital compression and coding of continuous-tone still images: Requirements and guidelines

ISO/TR 12033:2009 Guidance for the selection of document image compression methods

[1] This guide is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.46 on Cell Signaling.

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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.

2.3 *Other Documents:*

SWGDE/SWGIT Glossary SWGDE and SWGIT Digital & Multimedia Evidence Glossary, updated June 8, 2012[4]

U.S. Food and Drug Administration (FDA) Guidance, Technical Performance Assessment of Digital Pathology Whole Slide Imaging Devices[5]

European Machine Vision Association (EMVA) Standard 1288 Standard for Characterization and Presentation of Specification Data for Image Sensors and Cameras[6]

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *CCD bias, n*—the minimum analog offset added to the signal before the A/D converter to ensure a positive digital output each time a signal is read out. **E2642**

3.1.2 *charge-coupled device (CCD), n*—a silicon-based semiconductor chip consisting of a two-dimensional matrix of photo sensors or pixels. **E2642**

3.1.3 *complementary metal oxide semiconductor (CMOS), n*—technology widely used to manufacture electronic devices and image sensors similar to CCDs. In a CMOS sensor, each pixel has its own charge-to-voltage conversion circuit, and the sensor often also includes amplifiers, noise correction, and digitization circuits. Due to the additional components associated with each pixel, the sensitivity to light is lower than with a CCD, the signal is noisier, and the uniformity is lower. But the sensor can be built to require less off-chip circuitry for basic operation. **E2642**

3.1.4 *dynamic range, n*—the ratio of the full well saturation charge to the system noise level. It represents the ratio of the brightest and darkest signals a detector can measure in a single measurement. **E2642**

3.1.5 *electron-multiplying CCD (EMCCD), n*—type of CCD that has a two-way readout register, that is, the shift register and the gain register, each with its own output amplifier. When the charge is read out through the shift register, the detector works like a standard CCD detector, and when the charge is read out through the gain register, it undergoes charge amplification as a result of a different electrode structure embedded underneath the pixels of this register. **E2642**

3.1.6 *exposure time, n*—the length of time for which a CCD accumulated charge. **E2642**

3.1.7 *fluorescence, n*—the emission of radiant energy from an atom, molecule, or ion resulting from absorption of a photon and a subsequent transition to the ground state without a change in total spin quantum number. **E131**

3.1.8 *frame, n*—one full image that is read out of a CCD. **E2642**

3.1.9 *full well capacity, n*—the maximum number of photoelectrons that can be collected on a single pixel in the image area or in the horizontal register of a CCD. It is typically specified in terms of number of electrons. **E2642**

3.1.10 *irradiance, E, E_e, n*—the radiant flux incident per unit area. **E284**

3.1.11 *lossless compression, n*—compression in which no data are lost and all data can be retrieved in their original form. **SWGDE/SWGIT Glossary**

3.1.12 *lossy compression, n*—compression in which data are lost and cannot be retrieved in their original form. **SWGDE/SWGIT Glossary**

3.1.13 *photobleaching, n*—loss of emission or absorption intensity by a sample as a result of exposure to optical radiation. **E2719**

3.1.14 *pixel, n*—abbreviation for picture element. The smallest unit in an optical device in which charge is collected as a signal. CCD detectors typically have 26 µm square pixels; however, pixel sizes of 8, 13, 16, and 20 µm square are also available. **E2642**

3.1.15 *radiant energy, n*—energy transmitted as electromagnetic radiation. **E284**

3.1.16 *radiant flux, Φ, n*—the time rate of flow of radiant energy; radiant power. **E284**

3.1.17 *region of interest (ROI), n*—user-defined portion of the image area in which data will be acquired. The remainder of the image area will be discarded. **E2642**

4. Summary of Guide

4.1 Wide-field fluorescence microscopy is an optical imaging technique that relies on illumination of the entire field of view of a fluorescence microscope and simultaneous detection of the emitted fluorescence from all or a sub-region of the field of view using a camera. The emitted fluorescence can be measured as an intensity value in fluorescence microscopy, which is computed by summing together the intensity values from a group of individual pixels in a digital image acquired using a digital camera, such as a CCD, sCMOS, or EMCCD. A relative intensity measurement (RIM) is determined as the ratio of one intensity measurement to another, the result of which should be an accurate estimate of the ratio of the irradiance from part or all of a specimen to the irradiance from part or all of the same or another specimen.

4.2 The quantitative comparison of RIMs can be compromised or invalidated by many possible factors including the non-uniformity of intensities across the field of view of the microscope, the presence of an offset in the pixel values in the recorded digital image, the intensity signals in the image exceeding the linear dynamic range of the camera, or the inaccurate recording of the pixel values in image data files due to factors such as a lossy compression operation or unexpected modification of the pixel bit depth when saving each file. The quantitative comparison of RIMs can also be compromised by low signal-to-noise ratio of the measured light intensities or instability in the optical power of the illumination source.

[4] Available from Scientific Working Group on Imaging Technology (SWGIT), http://www.swgit.org

[5] Available from U.S. Department of Health and Human Services, Food and Drug Administration (FDA), Center for Devices and Radiological Health (CDRH). https://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/UCM435355.pdf. You may also send an e-mail request to CDRH-Guidance@fda.hhs.gov to receive a copy of the guidance. Please use the document number 1400053 to identify the guidance you are requesting.

[6] Available from European Machine Vision Association. http://www.emva.org/standards-technology/emva-1288/.

4.3 This guide provides a list of corrections and normalizations that are required so that RIMs can be accurately compared within and between images. This guidance document also includes a list of references to peer-reviewed, published methods that can aid in the qualification of instrument performance in Appendix X1. The collection of these resources should be useful in the design of robust cell-based assays that used quantitative fluorescence microscopy for data collection.

5. Significance and Use

5.1 *Overview of Measurement System*—Relative intensity measurements made by widefield epifluorescence microscopy are used as part of cell-based assays to quantify attributes such as the abundance of probe molecules (see ASTM F2997), fluorescently labeled antibodies, or fluorescence protein reporter molecules. The general procedure for quantifying relative intensities is to acquire digital images, then to perform image analysis to segment objects and compute intensities. The raw digital images acquired by epifluorescence microscopy are not typically amenable to relative intensity quantification because of the factors listed in 4.2. This guide offers a checklist of potential sources of bias that are often present in fluorescent microscopy images and suggests approaches for storing and normalizing raw image data to assure that computations are unbiased.

5.2 *Areas of Application*—Widefield fluorescence microscopy is frequently used to measure the location and abundance of fluorescent probe molecules within or between cells. In instances where RIM comparisons are made between a region of interest (ROI) and another ROI, accurate normalization procedures are essential to the measurement process to minimize biased results. Example use cases where this guidance document may be applicable include:

5.2.1 Characterization of cell cycle distribution by quantifying the abundance of DNA in individual cells (**1**).[7]

5.2.2 Measuring the area of positively stained mineralized deposits in cell cultures (ASTM F2997).

5.2.3 Quantifying the spread area of fixed cells (ASTM F2998).

5.2.4 Determining DNA damage in eukaryotic cells using the comet assay (ASTM E2186).

5.2.5 The quantitation of a secondary fluorescent marker that provides information related to the genotype, phenotype, biological activity, or biochemical features of a colony or cell (ASTM F2944).

6. Measurement Bias

6.1 Sources of bias in relative intensity measurements are listed below:

6.1.1 *CCD Bias*—The detectors used in widefield fluorescence microscopy are typically scientific complementary metal oxide semiconductor (sCMOS) sensors, charge-coupled devices (CCDs), electron multiplying (EM)-CCDs, or similar types of arrayed photodetectors (**2**). Regardless of the detector, the recorded digital signal in the absence of incident light, called the CCD bias (a.k.a. bias current offset or dark counts), is often not zero. The CCD bias is an offset that is added to each pixel in the digital image. Accurate determination of the value of the CCD bias is critical as it will need to be subtracted from the raw image.

6.1.1.1 A CCD bias that is less than zero is problematic. Digital images are typically saved in files that store only positive integer values. If the CCD bias is less than zero, an unknown offset is subtracted from each pixel value and relative intensity comparisons will not be possible.

6.1.2 *Linear Dynamic Range*—Images of the specimen under evaluation must be collected with the signal within the linear dynamic range of the detector. Signals that are at or below the noise floor of the camera will not be detected. Similarly, signals that are above the detector saturation are no longer in the linear range of the camera and cannot be used in relative intensity evaluations.

6.1.3 *Non-Uniform Field*—The intensities measured from a uniformly fluorescent sample are typically not uniform across the field of view. Field non-uniformities can arise from many factors, including non-uniform illumination, vignetting, and non-uniformities in the detector (ISO 13653 and (**3, 4**)). This means that the measured fluorescence intensity is dependent on its position within the field of view. If measurements are to be made in a region of the field of view with uneven illumination, a flatfield correction should be applied. An appropriate flatfield field correction will result in measured intensities that are not dependent on their location within the field of view and can be compared.

6.1.4 *Save Raw Images or Use Lossless Compression*—Many of the software packages that are used for controlling image capture from digital cameras offer the opportunity to save images in a lossy compression format (e.g. jpeg). This form of compression can alter the intensity data in a non-linear fashion, leading to unpredictable biases in relative intensity measurements. It is best to save raw, non-compressed image data or a lossless compression format (e.g. tiff) for images that are intended to be used to make relative intensity measurements. Additional modifications to the pixel values of an image can occur due to unexpected settings on the image analysis software. For example, bit truncation or bit depth conversion can occur on a saved image. It is worthwhile to evaluate the histogram of pixel intensities on the saved image and compare it to the histogram of the collected image to ensure the image is appropriately saved. A good resource for information on lossless and lossy image compression formats can be found in ASTM E2825-12, ISO/TR 12033:2009, and ISO/IEC 10918-1:1994.

7. Normalization Strategies for Sources of Bias

7.1 *CCD Bias*—To measure the CCD bias, a dark frame must be collected in the absence of illumination incident on the detector. This estimate for the CCD bias will only be accurate for the detector settings for which the image was taken. If any detector settings are changed, such as the temperature, gain, or binning, the CCD bias estimate may no longer be valid and may need to be remeasured. The CCD bias may change over the course of a data acquisition run caused, for example, by the

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

camera reaching a steady-state operating temperature during start up. Therefore, consideration should be given to measuring the CCD bias at the beginning and end of a data acquisition run to assure that no change in the CCD bias has occurred.

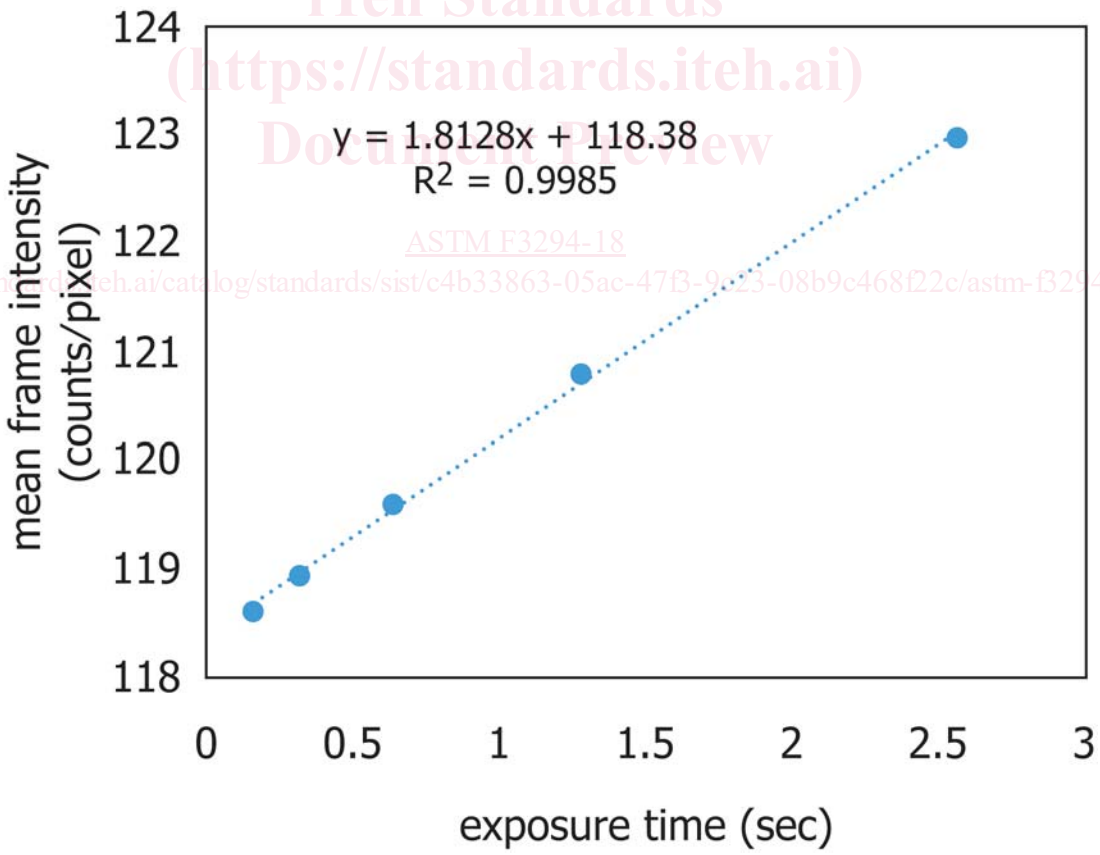
7.1.1 A simple approach for estimating the CCD bias is to turn off the microscope epi-fluorescent illumination and acquire at least 5 images at the same exposure time and same microscope settings (objective, filter sets, etc.) as used for the quantitative fluorescence images of the samples.

7.1.2 A more general approach is to turn off the microscope epi-fluorescent illumination and acquire at least 5 images at 5 or more exposure times. Plotting the mean intensity for each dark frame versus exposure time can reveal the presence of background light that is incident on the detector. A regression analysis of the mean intensity versus exposure time can be used to estimate the CCD bias value for any exposure time. An example of the data and analysis that can be used to estimate the dark frame counts is shown in Fig. 1.

7.1.3 A careful measurement of local background in the image of the sample is another approach that can be used to subtract the CCD bias. In this case, the mean pixel value of pixels near the object(s) being measured are used as an estimate for the background that should be subtracted before a RIM comparison is performed. The local background measurement would include the offset associated with the CCD bias as well as any background intensity coming from the sample such as fluorescence from the substrate or the media surrounding the cells.

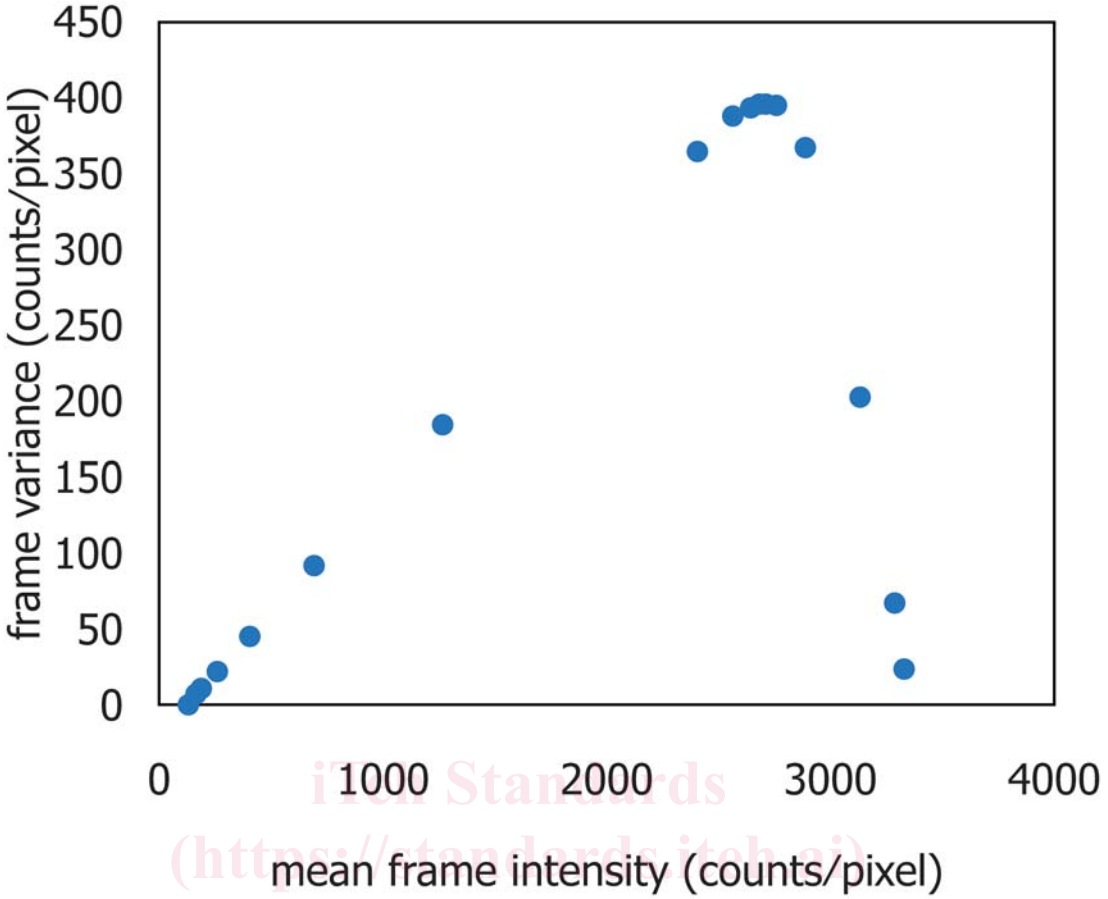
7.2 *Linear Dynamic Range*—Electronic image sensors are expected to be linear over a limited range. The upper bound of the linear dynamic range, saturation, can be determined by acquiring images from a uniformly fluorescent specimen at increasing exposure times (**5**). By taking two identical image frames and subtracting them pixel by pixel, the resultant image produced is composed only of noise related to the intensity measurement by each pixel. As illustrated in Fig. 2, plotting the mean frame intensity versus the pixel variance can be used to identify the intensity value at which the sensor begins to saturate. The pixel variance increases with the mean frame intensity until saturation is reached. On microscopes equipped with transmitted light illumination a similar analysis can be performed using light from the transmitted light source instead of fluorescence emitted from a uniformly fluorescent specimen.

7.2.1 It should be noted that saturation can occur below the maximum pixel range of the camera. As an example, for an array detector that records each pixel value as a 12-bit integer each pixel value will range from 0 to 4095. The recorded pixel



NOTE 1—In this example the mean intensity from a single frame at each time point is used. Least squares regression of the mean intensities is used to estimate the CCD bias = 118.38 counts. The data shown in the above plot is take from Halter et al. (**5**), where further details describing the data acquisition can be found.

FIG. 1 Example Plot of the Mean Intensity for Each Dark Frame Versus Exposure Time



NOTE 1—Images were acquired from a stably fluorescent glass at increasing exposure times. As the intensity increases, the pixel variance increases at first, reaches a maximum, then decreases. The data shown in the above plot are taken from Halter et al. (**5**), where further details describing the data acquisition can be found.

FIG. 2 Example Plot of the Pixel Variance in Each Frame versus Mean Intensity

value may not be proportional to the incident light intensity over this entire range as the response of the detector becomes non-linear at values less than 4095.

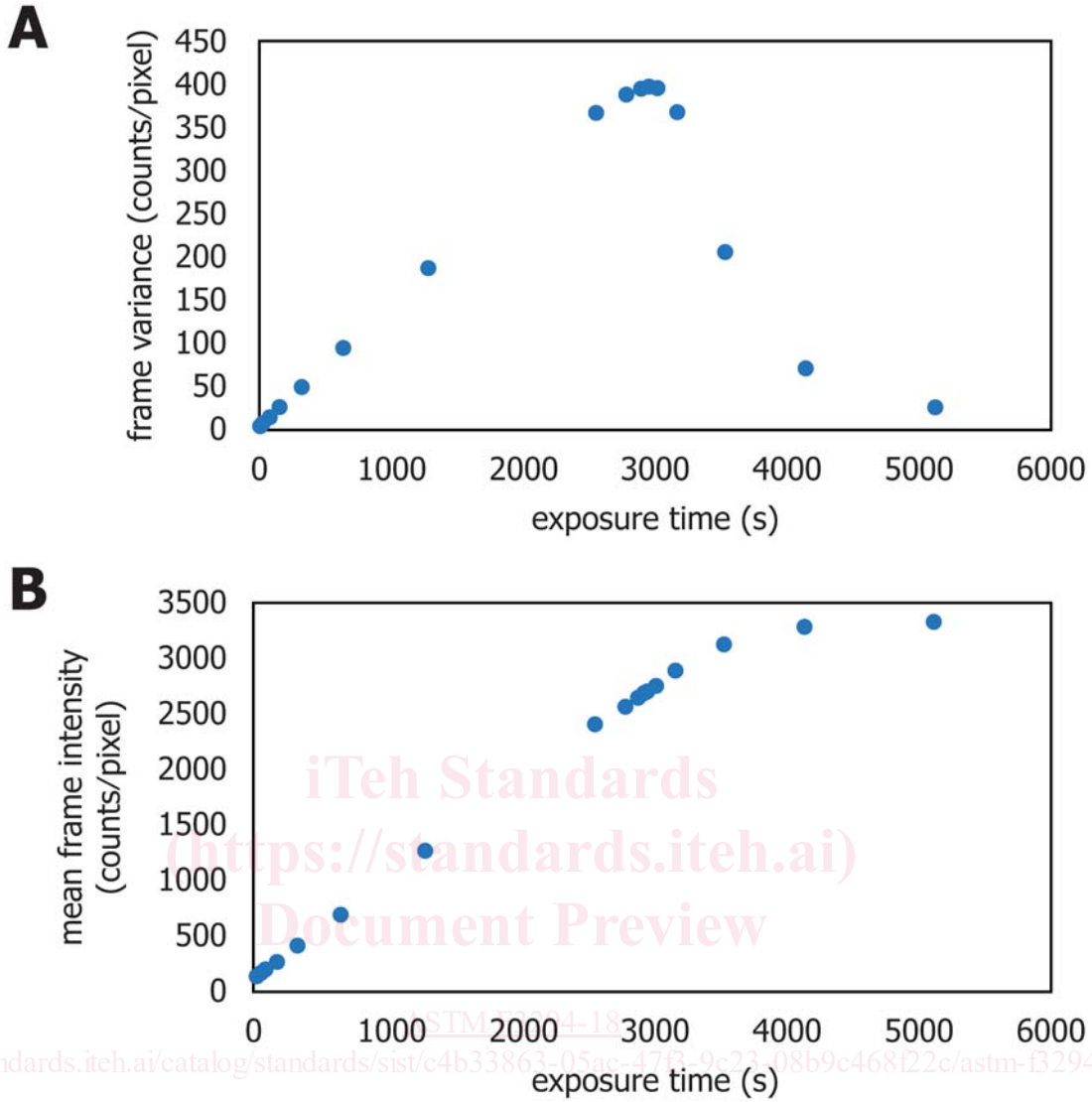
7.2.2 This method for determining the saturation of a digital camera attached to a microscope is very similar to typical approach (the photon-transfer-technique) that is used for determining image sensor and camera saturation that occurs when the well is filled to capacity (**6, 7**), and is part of the methodology of EMVA Standard 1288.

7.2.3 CCD and sCMOS detectors are known to exhibit a sharp decrease in the image variance when the sensor reaches full well capacity. This behavior can provide a well defined intensity level at which the detector saturates. An example of the sharp decrease in variance is demonstrated with the data in the plot in Fig. 2. The saturation can be defined as the maximum of the pixel variance versus intensity plot.

7.2.4 Plotting intensity versus exposure may also be an acceptable method for determining saturation. The plot takes on a very similar shape (Fig. 3A) to the mean frame intensity versus variance (Fig. 2). It is straightforward to determine the saturation point where the curve plateaus. Fig. 3B demonstrates that the saturation point for the full well capacity can be overestimated if the mean frame intensity versus exposure time is used. Both plots in Fig. 3A and Fig. 3B used data from the same experiment, so it is clear that the analysis method in Fig. 2 or Fig. 3A is superior.

7.3 *Non-Uniform Field*—The ideal correction for a non-uniform field would yield intensity measurements that are independent of an object's location in the field. Therefore, any correction scheme can be evaluated by moving the sample to different locations and measuring the intensity of the same object (**8**). During such an evaluation, one must be aware that photodegradation (i.e. photobleaching) of the sample may change the fluorescence emission from the sample. Measurements that demonstrate a small level of photodegradation can be used to support claims that the flatfield correction scheme removes biases due to non-uniform field effects.

7.3.1 A straightforward approach for estimating the non-uniformity across the field is to image the fluorescence emitted from a uniformly fluorescent reference sample. Imaging of the uniformly fluorescent reference sample must be performed under the same imaging conditions as the sample, since any change in the optical path, spectral filters, and detection sensor



NOTE 1—(A) As the exposure time increases, the variance increases at first, reaches a maximum, then decreases.

NOTE 2—(B) As the exposure time increases, the mean intensity increases, then gradually plateaus at a mean intensity in the range of 3000 to 3300 counts/pixel.

NOTE 3—The data shown in the above plot are taken from Halter et al. (**5**), where further details describing the data acquisition can be found. All images were acquired from a stably fluorescent glass at increasing exposure times.

FIG. 3 (A) Example Plot of the Pixel Variance in Each Frame versus Exposure Time, (B) Example Plot of the Mean Pixel Intensity in Each Frame versus Exposure Time

may change the flatfield correction. Candidate reference materials for estimating the flatfield correction include, but are not limited to:

7.3.1.1 High concentration solutions of sodium fluorescein for green fluorescence emission, Rose Bengal or Acid Fuchsin for red fluorescent specimens, or Acid Blue 9 for far red fluorescent specimens (**3**, **9**).

7.3.1.2 Fluorescein embedded in a thin polymer. This thin polymer sample is used to quantify the photobleaching rate at each location of an imaged field, which in turn permits computation of the flatfield correction (**4**).

7.3.1.3 Solutions of 7-dimethylamino-4-methylcoumarin, oxacarbocyanine (diO), indo-carbo cyanine (diI), and indodicarbocyanine dissolved in dimethyl formamide (**10**) in a glass bottom dish mounted on a depression slide.

7.3.1.4 Fluorescent plastic slides (**11**).

7.3.1.5 Fluorescent silver nanoclusters embedded in glass (**12**).

7.3.1.6 Fluorescent polymeric material (**13**).

7.3.2 Implementation of the empirical methods above for estimating the flatfield correction need to be optimized by adjusting the camera exposure time to use the full dynamic range of the camera without saturation. Acquiring several images from different fields and averaging the images in the image acquisition/processing software can reduce the effect of minor artifacts (e.g. from debris) in the real sample and