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# A Standard for Calibration and Shading Correction of a Fluorescence Microscope

**Michael A. Model and Janis K. Burkhardt***
Department of Pathology, University of Chicago, Chicago, Illinois

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**Background:** Numerous applications of fluorescence microscopy require quantitation of signal intensity in reproducible units. Two problems must be overcome to achieve this goal. First, due to various instrumental factors, the same sample imaged on two microscopes or even on the same microscope at different times may produce highly divergent readings. Second, because of shading, some areas within the same field may appear brighter than others despite the same amount of fluorophore. The first type of variability requires calibration using a sample of reproducible fluorescence yield; to correct for shading, a uniform fluorescent field is needed.
**Methods:** Standard slides were prepared by placing several microliters of 10%–50% w/v fluorescein or rhodamine between a coverglass and a slide. They were used to perform shading correction and normalization under a variety of imaging conditions.
**Results:** Concentrated fluorophores produced a uniform fluorescent field of moderate and reproducible brightness. By expressing the staining of a biological object in the units of standard slides, identical results were obtained irrespective of the imaging conditions or the microscope used. We compared shading correction based on concentrated fluorescein with two other standards. Concentrated fluorescein resulted in the best equalization of the field.
**Conclusions:** Standardization of fluorescent images can be achieved by normalizing them to the image of a concentrated solution of a fluorophore. Due to its simplicity and efficiency, this method can be used in clinical analysis as well as in routine laboratory practice. Cytometry 44: 309–316, 2001. © 2001 Wiley-Liss, Inc.

**Key terms:** fluorescence microscopy; standards; shading correction; sodium fluorescein; rhodamine 6G

Measurement of fluorescence intensity by means of microscopy has been used widely in medicine and research (1). However, despite enormous technical progress in all aspects of fluorescence microscopy, no generally accepted scale for signal quantitation has been developed. This shortcoming limits the use of fluorescence microscopy in areas where standardization is essential, such as medical diagnosis (2–5). In research applications requiring quantitative image analysis, the lack of appropriate standardization techniques hinders the direct comparison of data obtained at different times or on different instruments. Similar concerns apply to automated imaging, which is being used increasingly for cell screening and characterization (6–9). Here, uniform units of fluorescence intensity are useful in establishing parameters for analysis (e.g, to discriminate between positive staining and background fluorescence).

The signal from a digital camera is measured in gray levels, which are related to the number of photons reaching the detector. Such analysis yields consistent results only when all the components in the light path remain the same. When a sample is moved to another microscope that uses different optics and a different light detector, the measured number of gray levels is likely to change. Even on the same microscope, the illumination of the sample can be highly variable and the performance of the filters and the camera may change with time. Correction for this variability (calibration) can only be done with a sample whose fluorescence yield is constant. Several fluorescent standards have been described for this purpose. Fluorescent beads (10–13) or glutaraldehyde-fixed autofluorescent cells (14) have been used, but their discontinuous nature prohibits a straightforward correction procedure. Of continuous standards, uranyl glass, which is no longer available commercially, was shown to have reproducible brightness (15,16). Standards based on the fluorescent polymer, Spectralon, are spatially nonuniform (17); the same region of material has to be used every time, which precludes its use by more than one group of investigators. Dilute solutions of fluorophores placed directly between a coverslip and a slide are difficult to image due to bleaching and a variable depth of liquid. By placing the fluorophore solution in a hemacytometer (14) or in a specially de-

*Correspondence to: Janis K. Burkhardt, Department of Pathology, University of Chicago, 5841 S. Maryland Avenue, MC1089, Chicago, IL 60637.
E-mail: jburkhar@uchicago.edu

signed chamber (15,18,19), these problems can be alleviated. Even so, these standards may suffer from uncertainty in imaging a deep specimen, whether liquid or solid (1,17,20). Use of capillaries filled with a fluorochrome is discussed by Rost (1). Despite certain merits of this method, it has not become popular, probably because of the need for special equipment.

The other aspect to microscope standardization is correction for heterogeneity of illumination and detection within one single field (shading or flat field correction). Both computational (21–25) and experimental questions (13,20,26,27) of shading correction have been considered in the literature. The easiest solution to shading correction is to divide the image of interest by the image of a uniform fluorescent sample (after subtraction of the background). Many imaging programs, such as Metamorph, Isee, and Slidebook, are equipped with a routine to perform this operation. In each case, however, accurate shading correction requires the availability of a suitable standard. Presumably, the same continuous samples that are used for calibration can also be used for shading correction. To our knowledge, little data have been published on the efficiency of flat field standards applied to biological samples. Lockett et al. (13) did a comprehensive study of shading correction. However, they did not use a continuous sample, but rather fluorescent beads imaged at different locations followed by extensive image analysis. A simpler and faster procedure would be desirable.

We have found that it is possible to use a dye solution on a regular slide if the optical density of the solution is sufficiently high (28). In such a solution, the light penetrates only through a thin layer of liquid. For instance, if the extinction coefficient of fluorescein at 490 nm is 70,000 $M^{-1}cm^{-1}$, a 1-M solution will absorb completely the light within less than 1 μm. This fact makes the total depth of the sample less critical. Furthermore, bleached molecules in the illuminated layer are replaced rapidly by new molecules so that the sample can sustain prolonged illumination without a decrease in fluorescence. Because fluorescein solutions at high concentrations are susceptible to self-quenching by the Forster energy transfer, the brightness of concentrated solutions is moderate and comparable to the brightness of many cellular fluorescent dyes. For example, the signal from a 40% w/v solution of fluorescein was characterized in terms of the equivalent surface density of unquenched fluorescein and was found to be, for a particular filter set, 7,800 molecules per square micrometer (28), a number on the same order of magnitude as the density of many cell surface receptors.

In this study, we tested the usefulness of concentrated fluorescein solutions as standards for calibration and shading correction. We analyzed the reproducibility of measurements, stability of solutions, and the effectiveness of image correction in comparison with other standards. For shading correction, we find that concentrated fluorescein performs better than a commercial fluorescent slide or diluted fluorescein placed in a deep chamber. Our results show that concentrated fluorescein solutions can be used conveniently and reliably for both calibration and shading correction. We describe an analogous solution that can be used as a standard for correcting red fluorescence measurements.

## MATERIALS AND METHODS

### Materials

Fluoresbrite yellow-green 3-μm beads (Polysciences, Warrington, PA) and PS-Speck yellow-green 0.17-μm beads (Molecular Probes, Eugene, OR) were used. DeltaVision fluorescent slides were purchased from Applied Precision (Issaquah, WA). Mouse anti-phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY) and goat anti-mouse antibodies conjugated to Alexa 488 and Alexa 594 were obtained from Molecular Probes. For the mounting medium, we used Fluoromount G (Southern Biotechnology, Birmingham, AL) or Mowiol 4-88 (Hoechst Celanese, Charlotte, NC). The human thymoma cell line, Jurkat E6, was cultured in RPMI supplemented with 10% fetal bovine serum and 1:100 of the glutamine-penicillin-streptomycin stock solution (all from Life Technologies, Rockville, MD). Human fibronectin was a gift from Dr. A. Morla (University of Chicago). All other reagents were from Sigma (St. Louis, MO).

### Preparation of Fluorescent Standards

To prepare solutions of fluorescein, 0.1 M $NaHCO_3$ was added (without pH adjustment) to dry sodium fluorescein. The solutions were vortexed until no visible undissolved particles remained. They were either passed through a 0.2-mm filter or centrifuged at 16,000 g for 1 min. When sodium fluorescein was mixed with $NaHCO_3$ at 0.05, 0.1, 0.25, 0.5, and 0.75 g/ml solvent, the resultant solutions were found to be 4.9% w/v (0.13 M), 9.4% (0.25 M), 22% (0.58 M), 39% (1.04 M), and 52% (1.38 M), respectively, as was determined by measuring their final volume. Rhodamine 6G was dissolved in methanol at 0.3 g/ml solvent and centrifuged to remove insoluble matter.

Coverslips (18 × 18 mm, Corning [Corning, NY] 1.5) and slides were cleaned before use by immersion in 5% nitric acid for 20 min and rinsing thoroughly with deionized water. Several microliters of a fluorescein or rhodamine solution were placed between a coverslip and a slide and sealed with nail polish. The volume was chosen such that the liquid would spread over the entire area of the coverslip; no special precautions were taken to prepare ideally uniform samples. Rhodamine solutions in methanol had to be placed quickly under a coverslip before evaporation occurred. In some experiments, DeltaVision fluorescent slides were used as a source of a uniform fluorescent field. For observation of diluted fluorescein, an open chamber designed for cell observation in fluorescent light (Bioptechs, Butler, PA) was filled with 1 ml of 100 ng/ml Na fluorescein in 0.1 M $NaHCO_3$.

### Preparation of Test Specimens

Three types of samples were used to test shading correction: beads, cells, and a film of protein. Beads were

dried on a coverslip, which was then mounted on a slide, either dry or in a mounting medium. Jurkat cells were allowed to adhere to a coverslip that was precoated with 20 μg/ml fibronectin. They were made fluorescent by fixation in 2% glutaraldehyde for 1.5 h. Fixed cells were mounted on a slide in Mowiol. A film of fluorescent protein was prepared by incubating a glass coverslip with a solution of 2 μg/ml IgG-Alexa 488 (approximately 1 μl/$mm^2$) for 1 h, then rinsing and drying. The film is expected to have an average thickness of less than 1 molecular layer ($10^4$ molecules per square micron) if 100% of IgG was adsorbed from the solution.

For calibration experiments, we used Alexa 488 or Alexa 594-stained cells. Jurkat cells were allowed to adhere to fibronectin-coated coverslips and fixed with 3% paraformaldehyde/phosphate-buffered saline (PBS) for 20 min. Following fixation, cells were permeabilized with 0.3% Triton X100 for 1 min. Staining was achieved by incubation with 0.5 μg/ml 4G10 anti-phosphotyrosine, followed by 1 μg/ml of either goat anti-mouse Alexa 488 or goat anti-mouse Alexa 594. All incubations and washes were done in PBS/saponin/gelatin as described previously (29). After the final wash, a drop of mounting medium was added and the coverslips were mounted onto glass slides.

### Image Acquisition

Most images were acquired with an Axiovert TV 100 microscope (Carl Zeiss, Thornwood, NY) equipped with a 100-W Hg lamp. A 12-bit Micromax 1300Y CCD camera (Roper Scientific, Tucson, AZ) and the shutters (Ludl Electronic Products, Hawthorne, NY) were controlled by Isee 4.3.15 imaging software (Inovision, Raleigh, NC) run on an O2 workstation (Silicon Graphics, Mountain View, CA). Some images of cells were recorded using a PXL 1400 CCD camera (Roper Scientific) mounted on an Axioplan microscope (Zeiss) and controlled by OpenLab 2 software (Improvision, Boston, MA). For reproducibility and stability studies, and in the experiments with beads, we used a 63/1.4 plan-apochromat oil objective, which provided an observation area of 127 × 101 μm; for cell visualization, we used a Plan Neofluar 20/0.5 air objective (observation area 400 × 317 μm). To image diluted fluorescein and fluorescein at 9.4% or higher, we used a 0.2-s exposure and no neutral density filters; with 4.9% fluorescein, either a neutral density filter or a shorter exposure was used. Rhodamine was imaged at 0.1 s with ND 0.6; for DeltaVision slides, we used 0.01 s and ND 2.0.

In order to ensure reproducible focusing on standard slides, which appear rather uniform, the fluorescence field aperture was closed and brought into sharp focus, then opened again. The shadow of the field aperture can be observed only on the thin layer of fluorescent solution nearest the objective because the light does not penetrate into deeper layers. Thus, when the shadow is sharp, the objective is focused precisely on the coverglass-solution interface. The same method was used, although with less precision, with DeltaVision slides and diluted fluorescein.

### Image Analysis

Image analysis was performed with either Isee or NIH Image 1.62 (downloaded from http://rsb.info.nih.gov/nih-image/). Images were calibrated and corrected for shading according to the formula

$$\text{Corrected} = 100 \times \frac{\text{Raw} - \langle\text{Blank1}\rangle}{\text{Standard} - \text{Blank2}} \qquad [1]$$

where the standard image is produced by averaging several acquired images of the sample used as a standard, ⟨Blank 1⟩ is the mean gray level of the areas between the cells, and Blank 2 is the image (or its average gray level if the image was uniform enough) of a suitable blank sample (e.g., $NaHCO_3$ containing no fluorescein) obtained under the same conditions as the standard. The factor 100 was chosen to ensure that corrected images would have their gray levels in a convenient range. Note that in the commonly used flat field correction formula, a variable multiplication factor is used so that the corrected images have the same average brightness as the corresponding raw images (21,22). That formula cannot be used for calibration. By using its modified form [1], both flat field correction and calibration can be achieved.

## RESULTS

### Stability of Solutions and Reproducibility of Standard Images

To be useful for calibration purposes, a standard must be highly reproducible. To determine the effect of concentration on reproducibility, we tested various concentrations of fluorescein solutions. In the range we used, less concentrated solutions are brighter than those that are more concentrated. The relative brightness of 52%, 39%, 22%, 9.4%, and 4.9% fluorescein was approximately 1:1.2:1.6:3.8:15 (detailed data not shown; Fig. 1). In each experiment, several samples (i.e., coverslips) were examined, and each coverslip was imaged many times to correct for lamp fluctuations, at each of several locations. The signal from each location, with fluctuations averaged, constituted one experimental point. In most cases, there was no major difference in the variability between locations within one sample and the variability between samples, so the data for all locations and all samples were combined. As shown in Table 1, more concentrated solutions gave the most reproducible measurements. We achieved repeatedly a reproducibility of 97%–98% with fluorescein at concentrations of 10% or higher. The low level of variation is attributed partly to slow fluctuations in the lamp (on the order of minutes). As with fluorescein, a concentrated solution of rhodamine also gave highly reproducible measurements. Unfortunately, we were unable to prepare sufficiently concentrated solutions of rhodamine in solvents other than methanol. That makes rhodamine more difficult to handle than fluorescein because of rapid evaporation of the solvent.

An important factor in obtaining good reproducibility is resistance to photobleaching. To test this, concentrated

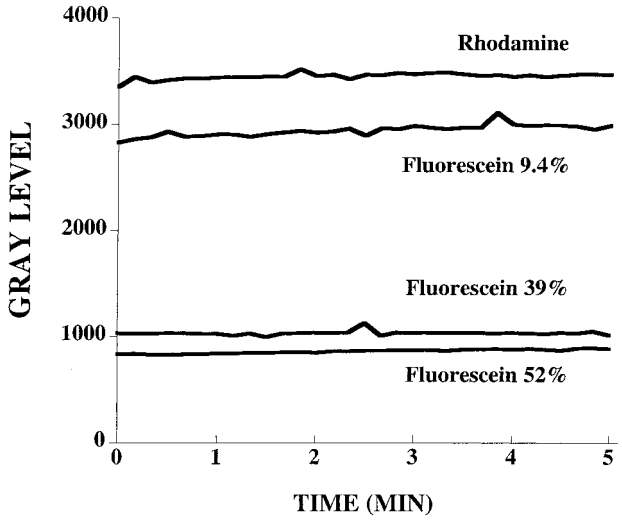


FIG. 1. Stability of standard slides to photobleaching. Standard slides prepared using the indicated concentrations of fluorescein or rhodamine were illuminated continuously by a 100-W Hg lamp through a 64/1.4 objective. Every 10 s, an image was recorded and the mean gray level was calculated.

fluorescein and rhodamine samples under a 63/1.4 objective were exposed to unattenuated light from the mercury lamp for several minutes. Every 10 s, an image was taken and its average gray level recorded. No decrease in fluorescence was noticeable for any of the samples tested (Fig. 1). Another important factor is the storage properties of the stock solutions used to prepare standard slides. To investigate this variable, concentrated fluorescein solutions were prepared on different days and kept in polypropylene microcentrifuge tubes in the dark, either at room temperature or refrigerated, and then analyzed on the same day. As shown in Table 2, there was no change in the fluorescence of samples prepared from solutions kept for up to 16 weeks. The temperature at which the solutions were kept also had no effect on fluorescence intensity (data not shown).

Table 1
*Reproducibility of Fluorescein and Rhodamine Slides**

| Standard solution (%) | Reproducibility (%) |
|---|---|
| Fluorescein 4.9 | 94 (4) |
| Fluorescein 9.4 | 97 (3) |
| Fluorescein 22 | 97 (4) |
| Fluorescein 39 | 98 (4) |
| Fluorescein 52 | 98 (3) |
| Rhodamine 30 | 97 (1) |

*Coverslips prepared using the indicated concentrations of fluorophores were imaged many times to correct for lamp fluctuations at three to five locations. For calculation of reproducibility, we included the data for all locations (after averaging fluctuations) and all coverslips. Reproducibility was defined as (1 − SD/mean)*100%. The number of times each experiment was repeated (on different days) is given in parentheses. The true concentration of rhodamine solution was not determined.

Table 2
*Stability of Fluorescein Solutions During Storage**

| Concentration of fluorescein (%) | 5 weeks | 10 weeks | 16 weeks |
|---|---|---|---|
| 4.9 | | | 99.9 |
| 9.4 | 97.2 | 100.1 | 97.9 |
| 22 | | | 100.6 |
| 39 | | | 100.0 |
| 52 | 101.1 | 99.8 | 101.6 |

*Solutions were prepared on different days and stored at room temperature in capped polypropylene microcentrifuge tubes in the dark. They were imaged on the same day, multiple times to minimize the effect of lamp fluctuations. The average intensity of each image was recorded and all the data for the same solution averaged. The numbers represent the ratio of the brightness of solutions stored for indicated periods to the brightness of a freshly prepared solution. The results indicate that fluorescence of concentrated fluorescein solutions does not vary significantly during at least 16 weeks of storage. They also show that our method of solution preparation is reliable.

## Shading Correction

Having found that the standard solutions give reproducible measurements over long periods, we tested their suitability for shading correction and calibration. Shading correction was tested on five specimens: autofluorescent cells in mounting medium, 3-μm polystyrene beads in mounting medium or dry, 0.17-μm dry beads, and a film of Alexa 488-labeled protein. To compare our fluorescein standard with other standards, we used, for a flat field source, a slide with 10% fluorescein, a chamber filled with diluted fluorescein, and a DeltaVision slide. No alignment of the lamp was done prior to the experiment; the darkest area in the field was found to be in the top right corner. Images were collected with each test sample (a cell, a bead, or a characteristically shaped area of adsorbed protein) in the top right corner, in the center, and in the lower left corner of the field. Neutral density filters were used to avoid bleaching while moving the sample over the field. Six objects from each sample type were imaged this way (four in the case of protein film). Shading correction was then performed on each set of sample images using each of the three standards and the ratio of the gray value in the corners to that in the center was calculated. A value of 1 indicates perfect correction. The results are shown in Table 3. In the raw images, the variation of object brightness across the field depends on the nature of the sample. Thus, it was not possible to use one flat field source to correct perfectly all possible images. Nonetheless, the 10% fluorescein standard gave good correction for all test samples, with residual nonuniformity within 10%. For four of five samples, 10% fluorescein gave the best correction of any standard we tried. The DeltaVision standard was slightly better with the dry polystyrene beads. Correction based on diluted fluorescein was the least accurate for all samples.

As an additional means of testing the efficiency of shading correction, a bead (from the sample containing 3-μm

Table 3
*Shading Correction Using Different Test Samples and Different Standards**

| | Uncorrected | 10% fluorescein | Diluted fluorescein | DeltaVision slide |
|---|---|---|---|---|
| Cells, Mowiol (814 ± 255) | | | | |
| L/C | 0.81 ± 0.10 | **0.94 ± 0.13** | 0.77 ± 0.10 | 0.90 ± 0.11 |
| R/C | 0.72 ± 0.05 | **1.00 ± 0.07** | 0.89 ± 0.06 | 0.90 ± 0.07 |
| 0.175-μm dry beads, (310 ± 103) | | | | |
| L/C | 0.73 ± 0.02 | **0.89 ± 0.04** | 0.69 ± 0.02 | 0.84 ± 0.03 |
| R/C | 0.59 ± 0.04 | **0.98 ± 0.05** | 0.78 ± 0.04 | 0.81 ± 0.04 |
| 3-μm dry beads, (451 ± 14) | | | | |
| L/C | 0.91 ± 0.01 | 1.07 ± 0.02 | 0.85 ± 0.01 | **1.03 ± 0.01** |
| R/C | 0.71 ± 0.02 | 1.08 ± 0.03 | 0.91 ± 0.01 | **0.94 ± 0.01** |
| 3-μm beads, medium (348 ± 30) | | | | |
| L/C | 0.84 ± 0.03 | **1.00 ± 0.03** | 0.80 ± 0.03 | 0.95 ± 0.03 |
| R/C | 0.68 ± 0.02 | **1.08 ± 0.03** | 0.88 ± 0.02 | 0.90 ± 0.02 |
| Protein film (1,232 ± 62) | | | | |
| L/C | 0.80 ± 0.01 | **0.93 ± 0.01** | 0.75 ± 0.01 | 0.89 ± 0.02 |
| R/C | 0.71 ± 0.02 | **1.01 ± 0.00** | 0.88 ± 0.01 | 0.90 ± 0.01 |

*The indicated objects (a cell, a bead, or an area of fluorescent protein film) were imaged at three locations: in the center of the field, in the top right corner, and in the bottom left corner. The signal (the mean gray level over the background) was determined. The mean ± SD for the value of the signal at the central location is shown in parentheses. The numbers in the columns represent the ratios of the signal from a selected object when it was moved to the top right corner (R/C) or to the bottom left corner (L/C) to the signal from the same object placed in the center of the field. For each sample type, the best correction is shown in bold. SDs were calculated based on six objects (four in the case of protein film) of each type.

beads in a mounting medium) was moved diagonally in small increments from the top right corner (the darkest area) toward the center. As it was brought into better illuminated regions, the brightness of the bead increased. A set of five images was collected as the bead was moved toward the center. Shading correction using each standard was applied to the images to compensate for the apparent increase in brightness. Figure 2 shows the amount of compensation achieved with each standard. A nearly perfect correction within the corner region was achieved with concentrated fluorescein, but not with the other two standards. Taken together, these results show that concentrated fluorescein standards are useful for shading correction under a variety of experimental conditions.

### Calibration of Images Obtained Using Changing Conditions

In the next set of experiments, the imaging conditions were made purposely different to mimic conditions that arise when samples are imaged at long time intervals or on different microscopes. Biological specimens containing cells stained for phosphotyrosine using Alexa 488 and Alexa 594-labeled antibodies were imaged under various conditions (Table 4). For some images, field illumination was changed by adjusting the collector lens on the lamp housing. In other cases, different optical filters were used. Moving the collector lens changed the overall brightness of the field and altered the distribution of illumination across the field. For instance, in Experiment 2, the values of gray levels (with background subtracted) in the flat field image varied initially over a 2.5-fold range (row 1) but over a 1.8-fold range after changing the position of the collector lens (row 2); defocusing of the collector lens also brought about a 2.1-fold decrease in the average brightness.

Four random fields were acquired for each condition. In each case, the images were corrected using a 10% fluorescein slide. The cells were identified by intensity thresholding, and the cumulative gray level was calculated for

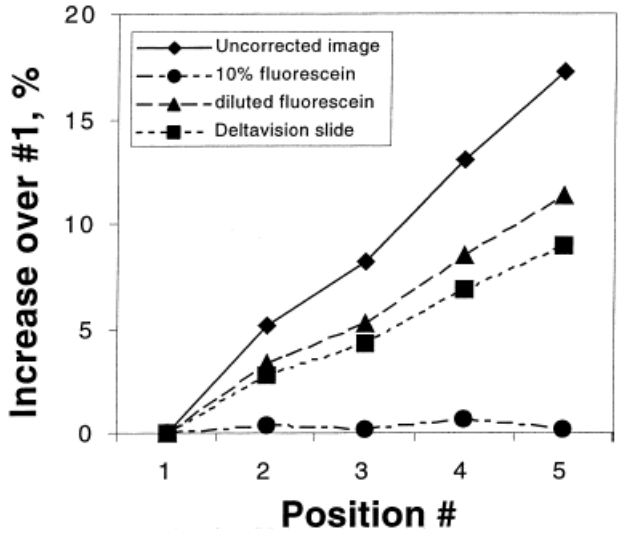


FIG. 2. Shading correction within a small heterogeneous area. A bead from a sample containing 3μm beads in mounting medium was imaged at five locations within the top right corner of an inhomogeneously illuminated field. Imaging started with the darkest location close to the very edge of the image (position 1) and ended with the brightest and the most interior (position 5). The vertical axis shows the increase in the bead brightness (with or without correction) relative to position 1. The brightness of the bead was determined by calculating the integral intensity from a region containing the entire bead and subtracting the integral intensity from a nearby background region of the same size. Raw images were corrected as described in Materials and Methods using each of the indicated standards to obtain the standard image (Formula [1]). The corrected values at each position are plotted for comparison with one another.

Table 4
*Use of Concentrated Standards to Calibrate Images Collected Under Varying Conditions**

| Microscope | Excitation (nm) | Emission (nm) | Experiment 1 Gray × $10^{-3}$, raw | Experiment 1 Gray × $10^{-3}$, corrected | Experiment 2 Gray × $10^{-3}$, raw | Experiment 2 Gray × $10^{-3}$, corrected |
|---|---|---|---|---|---|---|
| *Alexa 488* | | | | | | |
| 1 Axiovert | 493–507 | 515–555 | 41.7 ± 23.9 (85) | 17.4 ± 9.2 (92) | 78.4 ± 38.1 (201) | 16.8 ± 7.3 (202) |
| 2 Axiovert[a] | 493–507 | 515–555 | 68.7 ± 47.1 (132) | 17.6 ± 10.2 (134) | 38.4 ± 19.5 (257) | 17.9 ± 7.7 (249) |
| 3 Axioplan | 475–495 | >520 | 44.3 ± 25.3 (99) | 17.6 ± 9.1 (75) | 125 ± 65 (252) | 16.5 ± 7.4 (423) |
| 4 Axiovert | 450–490 | 500–550 | 157 ± 101 (90) | 9.11 ± 5.91 (134) | 188 ± 102 (183) | 8.62 ± 3.92 (152) |
| *Alexa 594* | | | | | | |
| 5 Axiovert | 556–580 | 590–650 | | | 81.7 ± 38.2 (208) | 3.59 ± 1.58 (241) |
| 6 Axiovert[a] | 556–580 | 590–650 | | | 133 ± 61 (193) | 3.61 ± 1.47 (228) |

*The experiment (sample preparation and imaging) was done twice. Jurkat T cells were labeled with anti-phosphotyrosine antibody and either Alexa 488 or Alexa 594. Each slide was imaged under the indicated conditions. For each condition, cells from four random fields were analyzed. The numbers represent the mean ± SD of the total gray level per cell (calculated as the mean gray level on a 12-bit scale multiplied by the cell area). The total numbers of cells analyzed in all fields are given in parentheses. The exposure time was kept at 0.2 s for all conditions except for Axioplan in Experiment 2 when it was changed to 0.5 s.
[a]The collector lens was moved to change the illumination of the field.

each cell. In the analysis of uncorrected images, the average background intensity was subtracted but no shading correction was applied.

Using images of green Alexa 488-stained cells under conditions where the lamp focusing was the only variable (compare rows 1,2 and 5,6), calibration using the 10% fluorescein standard worked well. For these conditions, the values before correction varied from $40 \times 10^3$ to $80 \times 10^3$. After correction, they fell in the range $16–18 \times 10^3$. A similar effect was achieved for red fluorescent cells. The same sample imaged on a different microscope with a longer exposure yielded, without correction, a value of $125 \times 10^3$ gray levels per cell (row 3, Experiment 2). Normalization brought it back to the expected $16.5 \times 10^3$. On that microscope, a slightly shorter excitation window was used, which also yielded efficient correction. However, a further shift in the excitation wavelength could not be compensated (row 4). These results show that normalization by fluorescein or rhodamine slides is highly effective when the difference is mainly due to illumination intensity and distribution.

## DISCUSSION

The problem of calibration and shading correction of images in microscopy has been receiving attention from researchers since the 1960s. Various solutions have been proposed, but there is still no consensus on the best approach. It may be that some of the successful solutions (13,15) required tools too elaborate to attract many followers. The described method of preparation of standard slides has the advantage of simplicity. Preparation of a slide requires no more than putting a drop of solution on a glass slide and covering it with a coverslip. Stock solutions are inexpensive and can be stored for a long time. We did not test their preservation beyond 16 weeks, but it is likely that the storage time can be greater. In any event, the low cost of Na fluorescein makes it easy to prepare fresh solutions.

In our tests, the reproducibility of the standard signal from concentrated fluorescein and rhodamine solutions was better than 95%. The glass must be clean, however, to ensure uniformity of the field. Occasionally, we observed a substantial transient change in the signal due to lamp instability. Therefore, to ensure correct calibration, we recommend repeated imaging of the standard several times during an experiment. To reduce noise in the image, we used averaging of several images. Alternatively, a low pass filter can be applied (22), which has a similar averaging effect. The difference between the two methods is that averaging is done over time in one case, and over space in the other. Averaging over time would be preferable only in the unlikely case of sharp intensity gradients in the flat field image.

The images of fluorescein solutions of different concentrations show identical distribution of brightness. Application of the correction formula based on 10%– 40% fluorescein (but not to the DeltaVision slide or diluted fluorescein) produces a nearly even field (data not shown). In other words, flat field images obtained using different concentrations of fluorescein can be converted into one another through shading correction. Theoretically, correction using any fluorescein concentration between 10% and 50% can be used for calibration; 5% fluorescein gives less reliable readings. We found that 10% solutions are less sensitive to dirt on the glass than more concentrated solutions. Some uncertainty may exist over the values of Blank 1 and Blank 2 in the correction formula. Therefore, it may be advantageous to use 10% fluorescein (the brightest) so that an error in choosing the background level would be minimized. When we used a 63/1.4 objective, the signal even from the most concentrated fluorescein solution was strong enough (typically, 500–800 gray levels over the background using a 0.2-s exposure). However, if a lower power objective is used, more concentrated solutions may not appear bright enough, compromising the precision of the measurement.

On the other hand, more concentrated solutions are less sensitive to slight deviations in fluorescein concentration and, on the average, showed a slightly better reproducibility with a 63/1.4 objective. Considering all these factors, we suggest using 10% solutions for shading correction. For calibration, more concentrated solutions can be used if their brightness is substantially higher than the background.

In testing shading correction, we corrected adequately for variations in intensity of as much as 40%. Our analysis of corrections between disparate imaging conditions (Table 4) indicates that variations up to twofold are tolerated. Because investigators who wish to perform quantitative analysis should never begin with a microscope that is grossly misaligned, this method should be adequate for shading correction in virtually all real experimental situations.

Our results with shading correction varied somewhat depending on the nature of the test sample. No single flat field standard can mimic perfectly the distribution of light in every sample. Despite this limitation, 10% fluorescein performed well with the majority of test samples. It was usually better than diluted fluorescein or the commercial plastic slide. DeltaVision fluorescent slides are also adequate for shading correction, although their extreme brightness may require density filters and short exposure times. However, in order to be useful not only for shading correction but for calibration as well, these slides must all have the same brightness. Because the uniformity of DeltaVision slides has not been tested by the manufacturer, they cannot be used as a universal calibration tool. Diluted fluorescein resulted in the least accurate shading correction. We conclude that concentrated fluorescein is an overall better standard for shading correction than deep translucent samples.

We investigated the ability of 10% fluorescein standards to correct for variability due to various factors: focusing of the lamp (which causes a change in both light intensity and distribution), a different microscope and camera, and different optical filters. We found that correction for the first two factors was quite successful, even though one of the microscopes used a shorter excitation filter. Moving the incident light further into the shorter end of the spectrum resulted in a preferential excitation of the concentrated fluorescein standard relative to the cellular stain Alexa 488 (whose spectral properties are similar to those of fluorescein). Application of the correction formula, which contains the standard image in the denominator, produced values that are too low. This finding can be explained by the fact that a fluorescent solution concentrated enough so that absorption of light becomes significant should have a broader excitation spectrum than a diluted solution. For these samples, even photons with a wavelength at the periphery of the absorption peak are likely to be absorbed and to stimulate fluorescence. Therefore, a concentrated standard is more efficiently excited by suboptimal wavelengths than a sample stained with a diluted dye. In addition to this, the spectrum of fluorescein is concentration dependent, although, to our knowledge, only concentrations much lower than used in our experiments have been investigated (30,31). To characterize more precisely the limits of acceptable filter mismatch, we attempted to measure the excitation and emission spectra of 10% fluorescein on a spectrofluorometer using a triangular cuvette (Starna Cells, Atascadero, CA). We were unable to obtain well-resolved peaks. Thus, further studies will be needed to characterize precisely the spectral range where calibration of a signal from fluorescein-stained cells is insensitive to the optical filters used. Until this is done, it is advisable to closely match the optical filters for the images to be calibrated.

## CONCLUSIONS

The use of concentrated solutions of fluorophores is a novel and effective way to correct for spatial heterogeneity of illumination and to standardize measurements with a fluorescence microscope. Standard samples prepared from concentrated fluorescein are reproducible, photostable, and their preparation requires no equipment other than a coverglass and a slide. The rhodamine standard performs much like fluorescein, with the caveat that it requires quick handling to avoid methanol evaporation. The stock solutions are inexpensive and stable during storage. The method can be used in any laboratory that uses digital analysis of microscopy images and it allows investigators to compare results obtained from different laboratories.

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