Design and construction of an optimal illumination system for quantitative wide-field multi-dimensional microscopy

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Abstract. A new modular transmission and fluorescence illumination system has been designed and built. The approach utilizes fiber optics to scramble the spatial intensity variations and digital monitoring of light intensity to allow accurate correction for inherent temporal instability. The illumination system has been characterized by a variety of methods and is shown to have an evenly filled objective back focal plane for maximal resolution in all three axes. The use of this illumination system in conjunction with a scientific grade CCD camera and pixel-by-pixel correction makes possible data of such high quality as to be only limited by the photon counting statistics. This illumination approach has proven to be particularly important for three-dimensional imaging coupled with image processing to remove out-of-focus information.

Keywords: Quantitative fluorescence microscopy, fiber optic scrambling.

1. Introduction

The last few decades have seen the light microscope evolve from a visual tool for investigating thin and stained samples into a quantitative analytical instrument which can reproduce faithfully three-dimensional information from large intact objects (Hiraoka et al 1991; for a recent review see Taylor et al 1992). While analytical spectroscopy based on photon counting detectors has been able to achieve extreme levels of sensitivity and accuracy, computerized microscopic video imaging has generally suffered from relatively poor accuracy. The introduction of scientific grade digital CCD cameras has eliminated the limiting factors of video cameras by offering a device with extremely low noise, high sensitivity, excellent linearity (0.025% or better), high resolution and superb geometric and temporal stability (Hiraoka et al 1987). Thus, high performance detectors are combined with the highly optimized optical components in current microscopes; however a major remaining limitation, for quantitative digital microscopy, is the illumination system.

Most microscopes use arc lamps as an illumination light source for both transmitted and fluorescence imaging. Arc lamps provide a source of intense, multi-wavelength light but suffer from inherent spatial and temporal instabilities (Chen 1990). The position of the arc discharge jumps erratically around the arc posts resulting in millisecond time-scale intensity fluctuations of the order of 5–10%. Not surprisingly, these fluctuations have proven to be difficult or impossible to eliminate. Furthermore, it is well known that the small intense light sources commonly chosen for microscopy have hot spots (e.g. Oriel catalog), of approximately 0.25 mm in size, which the collection optics image inhomogeneously. As a result, the back focal plane of the objective is poorly filled. The arc wander moves the hot spot around the objective back focal plane, leading to spatial changes of intensity on the image plane. The hot spots and the inhomogeneously filled objective back focal plane also cause a significant reduction in Z axis resolution, both for transmitted and fluorescence microscopy (Inoue 1986, Hiraoka et al 1990).

Wide field three-dimensional microscopy requires a space and time invariant optical transfer function (Agard 1984, Agard et al 1989) and that data be collected which are quantitatively consistent from section to section. This implies a stable illumination system. The conventional
arc-lamp-based digital fluorescent microscope systems constructed before 1990, generated three-dimensional images that suffered from the effects of source intensity fluctuations. Light source-derived aberrations can be clearly observed when examining X–Y, X–Z or Y–Z planes from reconstructed images. Because of the severe blurring in the Z axis (optical axis) produced by the point spread function, the deconvolution process can dramatically enhance section-to-section fluctuations. Thus a likely consequence of unstable illumination during data collection is the presence of abrupt intensity changes from one plane to the next when viewed along the optical axis. In addition, variations in illumination from one portion of a section to another caused by the wandering arc will also be magnified by the reconstruction process and will give rise to aberrations covering only a portion of the image within single X–Y planes. Intensity variations from section to section can be reasonably well corrected by appropriate algorithms after the data are collected. However, it has not proven practical to correct for spatial instabilities in the illumination. The combination of residual section-to-section and in-plane intensity variations leads to a readily observable degradation of resolution and image quality following extensive three-dimensional processing such as iterative deconvolution (Agard 1984, 1989).

Quantitative analysis in microscopy requires arithmetic operations on images, and the resulting data can be much smaller in magnitude compared to the original data. For example, to enhance the contrast of Nomarski pictures, background subtraction is performed. Calcium ion concentrations are determined from relatively small variations in ratio images. Such operations are often not limited by random shot noise but rather by temporal and spatial illumination instabilities. It is therefore necessary that the stability of the illumination system be better than 0.5%, the typical photon statistics noise figure for 12 bit data counts.

This paper describes a solution to both the spatial and temporal instabilities of the illumination system based on fiber optics technology. The light source is imaged onto a large 1 mm diameter moderately long quartz fiber. The fiber accepts the light in the entrance pupil and transports it with minimal losses and fully scrambles the spatial variations. It delivers the light at its exit pupil with a very smooth intensity profile and has a well defined numerical aperture. The remaining temporal variations from section to section are accurately normalized using a detector that samples the scrambled light as a function of time. The illumination system also provides for multiple light source inputs providing the flexibility for a diverse set of experimental protocols such as laser photo-bleaching or photo-activation of caged fluorochromes.

Previous reports describe the use of fiber optics for the trans-illumination light path, and the illuminator is available commercially (Ellis fiber optic light scrambler, Technical video, LTD., PO Box 693, Woods Hole, MA 02543). Improved Nomarski differential interference contrast enhanced images (Inoue 1986, Ellis 1985) and reduced coherent speckle effects in wide field laser illumination (Ellis 1979) were achieved. The present work differs from the above studies that use fiber optic light sources by the emphasis on long, high quality completely scrambling fiber optics in conjunction with achromatic lenses in order to achieve optimal transfer of light from the source to the object in fluorescence and transmitted microscopy.

2. Methods

2.1. The excitation light source unit

Figure 1 outlines the optical layout. A short arc, 100 W (Osram HBO/W2, Osram, Berlin, Germany) DC mercury lamp, in either a Zeiss or Olympus lamp housing containing a condenser, is coupled to the excitation light source unit. Immediately following is a KG1 (Melles Griot, Irvine, CA) heat filter. A Uniblitz 1 inch electro-mechanical shutter (VS22S1W1, Vincent Assoc. Inc., Rochester, NY) angled at 22.5° is spring mounted for vibration isolation. The shutter has a reflective coating which directs the light, in the closed position, into a light trap (type PL24, Klinger, Garden City, NY) with better than 10−4 extinction. Mounted adjacent are two 8 (or 10) position computer-controlled filter wheels for excitation wavelength selection and neutral density intensity attenuation (1 inch diameter, 6 mm thick filters

![Diagram](image)

(A) Arc lamp
(B) Heat filter
(C) Light trap
(D) Shutter
(E) Filter wheels
(F) Fiber coupling lens
(G) Quartz optical fiber
(H) Fiber optics input module
(I) Microscope

Figure 1. Schematic layout of the illumination system. The major mechanical and optical components are labelled.
from Omega Optical, Brattleboro, VT). The filter wheels utilize micro-stepping position control (Compumotor, Petaluma, CA). The filter wheels can be moved between adjacent positions within 0.18 second (or for the worst case of one-half revolution within 0.38 second), in a very smooth, accurate (one part in 50,000) rotary motion. The light is coupled to the fiber with a Fiber Input Collimator Type D-80HL (Mitsubishi Cable America, NY, NY) which is designed for use with laser beam fiber coupling. Many different lenses were tested for coupling the light into the fiber including high quality plan-apochromatic objectives, but these did not increase light coupling efficiency significantly. The overall light source-to-fiber distance was empirically optimized for overall light flux through the fiber.

The fiber optics cable selected is a 1 mm diameter UV grade quartz fiber (CS HPS/ST U-1000, Mitsubishi) with high power D-80/P ends. This fiber is designed for high power laser transmission in the deep UV to 1R range. Both ends are flat and highly polished. Fiber lengths, used on several microscopes, range from at least 2 metres (but also bent) to 15 metres.

According to geometrical optics considerations (Hecht 1990, pp 170, eq [5.60]) the number of reflections, \( N \), in such fiber are given by

\[
N = L \sin \theta / (D(\eta_i^2 \sin^2 \theta)^{1/2}) \approx 100 \text{ reflections/m}
\]

for \( \sin \theta \approx 0.1 \), where \( \theta \) is one-half the acceptance angle, the NA, and \( D \) is the diameter of the fiber, \( L \) is the fiber length in metres and \( \eta_i \) is the fiber core refractive index. Bending may be considered as a change in the average \( \theta \) (at the expense of some leakage of angles larger than the critical internal reflection angle), and can therefore increase the number of internal reflections by 2-3 fold, thus achieving the same degree of scrambling for a shorter fiber.

2.2. The Fiber Optics Input Module

Figure 1 illustrates the placement of the Fiber Optics Input Module between the end of the fiber and the microscope. An exploded view diagram of this module is shown in figure 2, and photographs of the components are shown in figure 3. The fiber optics is coupled by a NRC FPR-1C1 fiber optics positioner (Newport Research Corp. [NRC], Fountain Valley, CA), providing three axes of fine (80 threads per inch) positional adjustment. An achromatic lens (Spindler & Hoyer, Göttingen, Germany) \( f = 30 \) mm, collimates the fiber optics output beam. This lens optimizes the light collection and filling of the objective back focal plane but only partially fills the field of view although it generously covers the CCD image acquisition area. An optionally inserted beam expander, kinematically mounted on three tungsten carbide ball bearings and held in position by Neodymium Iron Boron magnets (#38428, Edmund Scientific, New Brunswick, NJ), increases the diameter of the illuminated field of view at the expense of light intensity. Two additional light source ports are provided. The selection is made by movement of kinematically mounted (again on ball bearings with magnets) adjustable mirrors (utilizing NRC Flexure Mirror Mount MFM-075) or dichroic mirrors. Finally the beams are sampled on the optical axis by a thin, 2 mm, UV-visible anti-reflective coated quartz beam splitter (Model S120 CVI Laser, Livermore, CA) which reflects approximately 0.5% of the incident light to a series of turret mounted pinholes; these are wavelength independent and reproducible.

Initially, a photon counting detector (SPCM-100-PQ, EG&G Electro-Optics, Vaudreuil, Quebec, Canada) was chosen to monitor the illumination intensity throughout each exposure. The detector is placed on the optical axis behind the pinhole, which is used to attenuate the incoming light. In order to compensate for the large differences between the intensities of the various excitation wavelengths and the wavelength-dependent response of the detector, two 3 mm thick absorption glass filters (UG3 and FG3, Melles Griot, Irvine, CA) are placed between the pinhole and the light detector. Photon
counting is gated by the digitally controlled shutter logic and thus integrates the light intensity during each exposure, as described below. The photon counter is connected to a counter board (CTM-05M, Keithley-Metrabyte, Taunton, MA) plugged into a PC. While a photon counter solution to the temporal instability is described here, less expensive silicon photodetectors are also being implemented.

The illumination system is designed so that the shutter controlling the illumination or excitation light is located before the fiber optics in order to scramble any shadows (especially prevalent at short exposure times) coming from the shutter. In the system, the light source shutter is dominant and thus sets the exposure time while the CCD shutter is opened before and closed after the light shutter. The function of the shutter sequence controller is to generate the requisite series of shutter control signals from a single input pulse. The CCD shutter is opened and the photon counter gate is activated immediately when the 5V DC pulse is applied from the CCD controller. When the CCD shutter reaches 90% opening, its SYNC signal becomes active, and is fed back to form a coincidence signal that allows the lamp shutter to open and to remain open as long as the controller signal is active. When the CCD controller goes inactive, the lamp shutter is first closed and then the CCD shutter is closed. Complete closure of the Uniblitz shutter requires a little less than 6 ms. Since there is no physical signal from the shutter to sense complete closure, an electronic 6 ms delay was included after closing the lamp shutter, before the CCD shutter was allowed to close. There is also a delay of 6 ms after closing the CCD shutter before the timing gate is deactivated. The actual exposure time is less than that given to the CCD controller. The actual minimum exposure is approximately 5 ms.

Alignment of the fiber optics system is straightforward. First the fiber exit pupil is focused and centered in the objective back focal plane by manipulating the Newport fiber optics positioner. It is assumed, on the basis of mechanical design, that the fiber and optics are coaxial with the microscope axis. Next the image plane is examined; the entire illumination module is translated to center the illuminated area. These steps are repeated. A mercury lamp change is accomplished by first replacement of the arc lamp followed by optimization, using the lamp housing translation and focusing.
Optimal microscope illumination

3. Results

3.1. Design considerations

A number of design parameters were considered for this fresh evaluation of the microscope illumination system. First, spatial intensity variations, in the object plane, had to be removed. Second, it was desirable to completely and homogeneously fill the objective back aperture for maximum resolution, using the full numerical aperture of the lens. Third, the temporal intensity fluctuations had to be determined and corrected. Fourth, it was important to preserve the field and aperture conjugated planes, all the way back to the light source, but at the same time making sure that the light was relayed with maximum efficiency and minimum spillout through the lenses and stops. In order to accomplish this task software ray-tracing optics design and simulation tools were used. These tools allowed selection of a unique position for a single achromatic lens, which imaged the fiber optic exit pupil onto and filling the objective back aperture fulfilling Köhler illumination principles.

As described in the methods, the light emerging from the lamp is gated by a computer controlled shutter followed by wavelength selection through the use of fast smooth computer controlled filter wheels and attenuated by neutral density filters, again computer controlled. The light is then imaged onto a polished end, high power, laser quality quartz fiber optics. The light emerges at the other end of the fiber optics into a flexible module that images the light onto the objective back aperture. The module, as seen in figure 2, allows for multiple light sources, easily selected, that permit a diverse choice of biological experimental approaches. For example, a second fiber optics could be used for microbeam UV caged fluorochrome activation (Mitchison 1989) or another port permits laser photobleaching experiments (Benveniste et al 1988). Flexibility in the biological experiments as well as future modification or insertion of other beam modulating optical components is provided by this design.

While this illumination is primarily designed for three-dimensional fluorescence microscopy, it was straightforward to modify this design and implement a transmitted light source (see figure 3) that also meets the same optical criteria.

3.2. Optimal exposure control

Every illumination system, especially those built into digital microscopes for biological experimentation, must contain a means of source illumination exposure control. Typical shutters, usually leaf shutters, are placed reasonably near imaging planes and with short exposures (<0.1 s), create shadows on the image due to the differential opening time of the centre compared to the periphery of the shutter (figure 4A). This is not acceptable because this effect is strongly dependent on exposure time and is imprecise due to mechanical variability. The current design corrects this problem directly. The light source shutter is placed behind the fiber optics, thus any shutter shadows are scrambled by the fiber optics. The detector shutter, usually the CCD shutter, is controlled digitally to avoid shutter shadows (figure 4B) by the simple expedient of opening the CCD shutter before, but closing it after the illumination shutter. This process is carried out by digital delay and shutter control electronics as described in the Methods. This same control electronics also gates the photon counter (or silicon detector) for determination of the integrated intensity of an imaging exposure. This allows for more precise data collection and decreased sample photodamage.

3.3. Quantitative measurement of the spatial stability of the illumination system

For correct illumination of the microscope, the incident light beam geometry has to fill the objective back aperture, and the illuminated field has to uniformly cover at least that part of the field imaged by the CCD. These conditions can be met by magnifying the fiber exit plane to fit the size of the observed field and matching the light cone to the angles accepted from the back of the microscope. For this purpose we examined, through the eyepiece of the microscope, the image of a 2 mW He–Ne laser beam directed through the epi-illumination port of the microscope. The desired parameters were determined by focusing, shifting and tilting the laser beam.

Figure 5 shows the objective back aperture focal plane for both fluorescence (5A,B) and transmission (5C,D) illumination. Placement of a mirrored slide at the object plane allows the image of the fiber optics end to be
Figure 4. Elimination of shutter shadow. Ratio of two images of a thin layer of dye solution taken at 10 and 0.02 s exposure times are shown (A) Shows the ratio using the previous unmodified instrumentation where the CCD shutter sets the exposure time and the illumination shutter opens before and closes after the CCD shutter. The bright periphery is indicative of the relatively shorter exposure time as compared to the centre due to shutter shadow. (B) Is the same ratio for the new instrumentation with illumination shutter behind the fiber optics, and CCD shutter opening before and closing after illumination shutter. The shutter shadow effect is totally eliminated. Scale for both panels (black to saturated white) (450–550 counts).

accurately focused and centred on the back focal plane. There is a very shallow gradient across the back aperture focal plane. Translation of the fiber shows its sharp edge in focus with the objective back focal plane. No substructure within the illumination field is visible even at high contrast. There are weak diffuse rings and a small spot visible in the back focal image. These features, however, do not translate with the fiber (and are not seen in the back focal plane images in fluorescence experiments) hence are likely to be weak reflections or other aspects of the microscope optics. One edge is slightly brighter than the other due to a very slight tilt of the achromatic collimating lens with respect to the optical axis just after the fiber. Recent illumination system designs have allowed for tilt correction. Very similar back focal plane images are seen with transmitted microscopy as shown in figure 5C, D.

To demonstrate directly that the current design can eliminate, to a high degree of precision, the spatial intensity variations due to arc wander, images were taken following deliberate misalignment of the arc position in the lamp housing. Figure 6 shows ratio images before and after arc misalignment which produced a 50% decrease of overall intensity. Arc misalignment was translated both up, figure 6A, and down, figure 6B. The resulting images are greatly stretched in contrast, and demonstrate that the total variations do not exceed ±0.5%. This establishes that a greater than 100-fold improvement in spatial intensity variation has been achieved (50% input intensity changes versus 0.5% changes of the output profile). The fiber optics illumination system utilizes a physically long fiber for proper scrambling of the light; at least two metres of fiber length is required to remove the characteristics of the fiber light launch system and minimize intensity variations arising from fiber flexing and bending (Oriel catalog, vol II, p. 403).

3.4. A comparison of Köhler versus critical illumination with fiber optics light source

Now that the light emerging from the fiber optics is so homogeneous and stable, a new look at Köhler versus critical illumination is possible. A geometrical ray tracing of these two types of illumination schemes is shown in figure 7. Study of this figure indicates, as is well known, the Köhler illumination is very extended at the image plane but critical illumination has a sharper, more intense distribution. Critical illumination has not been used because the source produced inhomo-genous, greatly extended Z axis components and an unstable profile of intensity. However, the illumination optics system, described here, makes it desirable to re-evaluate this issue. It is proposed that critical illumination now be utilized which will have the advantage of somewhat increased resolution in Z.
Figure 5. Objective back focal plane images. Epi-fluorescence Illumination: the back focal plane of a Zeiss Axiomat 100 ×/1.3 plan-achromat objective is photographed with the use of the Bertran lens. A mirrored slide is used and is viewed in focus. The wavelength is 546 nm. The image is photographed with Kodak 2415 film and developed with D-19 for extremely high contrast. Panel A shows the fiber optics centred in the back focal plane, while panel B shows the fiber slightly translated; note the sharply focused fiber edges that just fill the back aperture. There are very slight spots of dust and small immersion of oil–air bubbles visible. Transmission Illumination: the clear glass slide and coverslip sample is in focus in the bright field mode, and the back focal plane for 100 ×/1.3 objective is photographed. Panel C shows the fiber optics centred, while panel D shows the fiber translated with respect to the back focal plane.

(important for three-dimensional microscopy) as well as increased overall flux. As measured, critical illumination provides a small two-fold increase in light flux at the sample plane, compared to Köhler illumination. It will be necessary, however, to step the sample in Z to alter the focus instead of moving the objective as is currently done. That is, the stage, containing the sample, must translate accurately along the optical axis.

While it is planned to use critical illumination in the future, the current microscope design uses Köhler illumination. The experimental work in our laboratory emphasizes three-dimensional imaging. Thus, it is important to demonstrate minimal spatial intensity variations as a function of defocus. As shown in figure 8, using 100 ×/NA1.3 objective, the variations in the centre of the field caused by 10 and 50 microns defocusing above and below a given plane are much less than 1%, while those at the periphery of the field are approximately 1% and 5%, respectively. Further measurements (not shown) indicate that these variations are symmetrical above and below the focus.

3.5. Efficiency of light transmission through the illumination system

It is of interest to determine regions in the illumination path where light losses take place. A number of light flux measurements were made at different points along the light path in the microscope. Unfortunately, it is difficult to characterize absolute values since the measured
Figure 6. Fibre optics correction of spatial variations. The ratio of two images of a layer of dye solution, taken before and after misalignment of the arc position causing about half of the light to miss the fiber input is shown. Panel A is the image ratio of an image taken after the arc was shifted up divided by the aligned arc image; panel B is the same except that the arc was shifted down. The ratio images, displayed with very high contrast, demonstrate that the large spatial changes at the fiber input are effectively scrambled by the fiber. The relative light intensities at any point in the field varied no more than \( \pm 0.5\% \), indicative of the small deviations from perfect scrambling. The images are scaled from black to saturated white as follows: ratio scale for A: 0.548–0.553, ratio scale for B: 0.406–0.410. This determination utilized a fiber optics of 2 m in length; other implementations of our microscope-fiber optics illumination systems have fiber optics cables of 10–15 m for increased spatial scrambling.

Figure 7. Geometrical ray tracing through a microscope optical path showing Köhler and critical illumination. Rays emerging from the source positioned for critical and Köhler illumination (dashed and continuous lines respectively) are traced to the object plane using Gaussian optics approximations. The foci and distances are taken from Bradbury (1989). The area between two rays from one point on the ‘critical’ source is dotted and for two other rays emerging from the Köhler source the area is painted by crosses. Some of the same rays can be traced from both positions of the illumination sources. However, more rays are collected in critical illumination, and cross the object plane at a larger numerical aperture.
intensities depend on the lamp age, filter characteristics and most importantly the dichroic mirror. A summary of light energy transferred through the illumination system and the microscope is presented in table 1, for 6 typical excitation wavelengths in use for biology. First, an average of 10% of the light from a given wavelength window—UV to IR—makes it through the fiber (15 m long); this loss is essentially due to the fiber coupling. It is likely that only the mercury arc hot spot makes its way into the fiber; ray tracing simulations show large spherical and chromatic aberrations due to the collector condenser optics. Second, a reasonably large fraction of the light emerging from the fiber makes its way through the microscope into the objective. Values from 17% (for 365 nm) to 53% (for 580 nm) are measured but are highly dependent on the choice of dichroic mirror (table 1).

Table 1. The light intensity was measured at various points in the illumination path with a calibrated silicon detector (Newport Corp. Model 835 with a UV attenuator/detector Model 818). Six different filter-wavelength combinations are listed. First, the light intensity just after the wavelength filter was measured. Secondly, the light intensity just after the fiber optics was measured with the percentage (%) of the light traversing the fiber noted. Finally, the light intensity at the objective holder (but no objective) was measured. Two different dichroic mirrors were used: the 80:20 refers to achromatic beam splitter that reflects, into the objective, 20% of the incident beam and transmits 80% of the emitted fluorescent light. The % refers to the percentage of the light, measured at the end of the fiber optics, that arrives at the objective holder.

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Excitation wavelength (Å)</th>
<th>After filter before fiber optics (mW)</th>
<th>After fiber optics (mW) (%)</th>
<th>DAPI/Texas Red dichroic (mW) (%)*</th>
<th>80:20 beam splitter (mW) (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI</td>
<td>355</td>
<td>77.2</td>
<td>5.85 (7.6)</td>
<td>0.97 (16.6)</td>
<td>0.14 (2.4)</td>
</tr>
<tr>
<td>Ext. DAPI</td>
<td>405</td>
<td>72.4</td>
<td>5.67 (9.1)</td>
<td>0.52 (7.9)</td>
<td>0.44 (9.8)</td>
</tr>
<tr>
<td>FITC</td>
<td>485</td>
<td>51.5</td>
<td>7.00 (13.6)</td>
<td>0.49 (7.0)</td>
<td>0.66 (9.4)</td>
</tr>
<tr>
<td>Rhodamine</td>
<td>543</td>
<td>183.0</td>
<td>21.57 (11.8)</td>
<td>9.90 (53.9)</td>
<td>2.20 (10.2)</td>
</tr>
<tr>
<td>Texas Red</td>
<td>580</td>
<td>off scale</td>
<td>39.70 (11.8)</td>
<td>21.05 (53.9)</td>
<td>4.54 (10.2)</td>
</tr>
<tr>
<td>Cy5</td>
<td>647</td>
<td>13.5</td>
<td>1.85 (13.7)</td>
<td>0.038 (2.1)</td>
<td>0.23 (12.4)</td>
</tr>
</tbody>
</table>
Similar light loss measurements were made on a Zeiss Universal microscope using Zeiss filters and dichroic mirrors with the lamp directly coupled for comparison. Three times more light makes its way to the objective back focal plane at 550 nm (34 mW) on this microscope indicating that there are also large light losses in conventional fluorescent microscope illumination paths.

In order to compare the fluorescent intensity of directly coupled light sources with the fiber optics illumination system described here, light emitted from fluorescent beads was measured on the same microscope. Fluorescent intensity measurements were performed using standardized fluorescent beads (0.12 μm, Molecular Probes, Eugene, OR). The intensity of a bead in focus, reflecting the actual light flux density at the object plane has increased by about 30%; the average peak intensity for 73 beads for 0.8 second exposure was 2300 counts with direct lamp illumination as compared to 3100 counts [61 beads] with fiber illumination. This is taken to mean that light arriving via fiber optics has fewer aberrations, making for a more efficient passage through the objective.

This design places the excitation wavelength selecting filters before the long optical fiber. Previous studies without the use of fiber optics showed that every filter position produced a slightly different angle of illumination leading to spatial variations in the image plane. In addition, the angular dependence of the transmitted wavelength bandwidth caused a different spectrum at the periphery of the incident beam compared to the centre. These problems are eliminated by our design. However, fiber fluorescence must remain insignificant so that the background of the biological fluorescent samples is not raised. Autofluorescence of the fiber plus microscope was measured at the entrance of the objective lens for 6 wavelengths from 365 to 650 nm using a mirror slide. Typical values range from 8.3 x 10^-6 of the incident intensity (for 365 nm) to 5 x 10^-8 (for 650 nm); these numbers are very close to the extinction specifications of the filters themselves. Thus, the fiber does not contribute significantly to autofluorescence.

Quartz has a wavelength-dependence of the index of refraction which ranges from n_q = 1.475 for 365 nm down to n_q = 1.452 in the red (850 nm). The fiber numerical aperture depends on n_q and n_a, (refractive index of the cladding) as NA = (n_q^2 - n_a^2)^1/2 (Hecht 1990, p. 171, eq. 5.64). The fiber manufacturers list n_q = 1.452, n_a = 1.438 at 850 nm, which calculates to a NA = 0.201. For the UV (365 nm) the fiber NA may be as high as 0.203. These dispersion effects show up as a slight wavelength dependent illumination profile, and is taken care of by different CCD gain and offset correction files for each wavelength filter.

With CCD imaging devices, a pixel-by-pixel digital correction must be applied (Hiraoka et al 1987, Agard et al 1989) in order to normalize the variations in the sensitivity of each CCD pixel. Achieving a high degree of illumination spatial stability makes it possible to flatten and homogenize the field to a very high degree of precision. The direct consequence of the spatial stability is the ability to accurately measure and normalize for temporal fluctuations by sampling the illuminated flux at any point in the field. Actually, this precise digital correction serves to remove essentially all systematic instrumental effects, and produces images which accurately reflect the number and distribution of fluorophores in the sample limited only by photon statistics and the point spread function.

4. Discussion

The purpose of this paper is to demonstrate that it is possible to design and implement a generalized illumination system applicable for both transmitted and fluorescence microscopy, that fundamentally corrects spatial and temporal light source variations. As described in the results, this approach reduces spatial and temporal fluctuations to less than 0.5%, a goal and requirement for the quantitative analysis of biological image data. The resultant intensity data, at high magnifications and using large numerical aperture objectives (resolution ≈ 0.2 μm), approach the accuracy expected by the photon statistics. Our primary goal in this work was the implementation of an illumination system that would be ideal for the collection and subsequent quantitative analysis of three-dimensional images. Removal of the spatial and temporal light source instabilities has greatly improved the three-dimensional deconvolution process (Swedlow et al 1993) using the criterion of lower R-factor (see Agard et al 1981) as a function of iteration number. In addition, the improved data result in more rapid convergence and lower backgrounds. Examination of three-dimensional data from any perspective, both before and after deconvolution, shows smooth transitions between sections and within sections and not the obvious discontinuities found with using conventional illumination. It is anticipated that the use of critical illumination in conjunction with focusing by Z translation of the sample will additionally improve Z axis resolution (see, for example, Hiraoka et al (1990) for discussion).

A key feature of this illumination system is the inherent flexibility for different modes of illumination. Three ports for light input are provided, and more could be incorporated in the future. Current directions in biological research require multiple models of imaging. The ability to activate a caged fluorophore with a UV microbeam to create a fluorescently labelled region is likely to be an important tool; the second input port was designed to facilitate this approach. However, to avoid altering the illumination conditions, the sample needs to be translated in Z during focus or for three-dimensional
data collection. (A high precision, computer-controlled three-axis stage has been designed and built for this axis of the microscope, and is useful for laser photobleaching (or UV micro spot) experiments and other dynamic studies.

This illumination system is directly applicable to a variety of interesting experimental paradigms in modern biological microscopic research. The reduction of the spatial and temporal variations due to the light source to less than 0.5%, results in dramatic reduction of systematic errors, in the recorded image data. Microscopic methodologies requiring ratios (or differences) of images should now be able to provide considerably more accurate results, with a much higher confidence in the reality of small effects. In this category are studies on specific ions, voltage sensitive dyes or pH measurements on small cell organelles present at low concentrations. Another key area that absolutely requires a constant illumination source is fluorescence resonance energy transfer. Direct imaging energy transfer methodology is technically extremely demanding and has recently been implemented using the described illumination technology (Kam et al. 1993). It is also conceivable that other more demanding approaches will be possible, for example imaging resonance Raman spectroscopy (Marriott et al. 1991) and fluorescence nanosecond time resolved microscopy. In all these applications, it is very desirable to extend the imaging modality to three-dimensions as well as the time domain.

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