TN424: A study of fluorescence standards confirms that OptiGrid confocal images are suitable for quantitative microscopy

Introduction

The OptiGrid converts the illumination system of a conventional wide field microscope to a structured light illumination system. Control of Grid movement and image processing of the raw structured illumination images by a computer system allow for the generation of images with only in focus detail (Neil et al 1997). This technique effectively confers confocal capabilities to conventional wide field microscopes (Puche 2005).

Other techniques that generate confocal-like images from wide field microscopes exist, such as deconvolution. In some cases these techniques can be used for accurate quantitative microscopy, such as iterative restoration. In other cases these techniques are known to be unsuitable for accurate quantitation, such as fast restoration.

The efficacy of the OptiGrid for quantitative microscopy is unknown. We have therefore acquired grid confocal images using known fluorescence intensity standards. Quantitation of the intensity of these images revealed that the OptiGrid is suitable for quantitative microscopy studies.

Materials and Methods

Acquisition

Fluorescence standards were prepared from an InSpeck Green (6µm) Microscope Image Intensity Calibration kit (Molecular Probes, Eugene OR. Catalogue Number I14785). Slides were prepared by drying 5µl of components C,D,E, F and G onto glass slides, and mounting in 10µl of mounting medium (component H). This produced intensity standards of 1.2% relative intensity (RI), 3.4% RI, 11% RI, 33% RI, and 100% RI.

Slides were imaged with a Zeiss Axiovert 200M microscope equipped with an Optigrid (Thales Optem), a Zeiss number 10 filter block, an Orca ERG CCD camera, and an Exfo 120 light source (Exfo). The microscope, camera, and Optigrid were controlled from an Apple G5 running Volocity 3.6 (Improvision). All the images were acquired for this study using a 63x apochromat (1.4NA) oil immersion lens (Zeiss).

Exposure times were set by using the Volocity “auto-exposure” function with the 100% RI slide mounted. The same exposure time was used for all the other intensity standards, no microscope, light source or camera controls were changed during the acquisition of an intensity standard series. The same exposure time was used for the acquisition of both grid confocal and grid wide field images, a different exposure time was used for the acquisition of true wide field images (removal of the Optigrid

from the illumination system allows more light to reach the specimen). The camera gain and offset were both at their minimal settings.

Volumes 8µm thick were acquired of the 6µm thick beads, ensuring that the whole of the beads were imaged. The step size was 100nm in depth. Each volume was composed of 81 z sections.

Analysis

A three dimensional region of interest (ROI) containing 7616 voxels (total volume 18.7µm^3) was drawn entirely within a bead. The mean intensity within the ROI was measured. This was repeated for every bead within a single field of view for each RI sample and the mean of the mean intensities was calculated using the Volocity measurement functions. The same ROI was used for all measurements, when moving the ROI between beads the position of the ROI was carefully checked in the XY, XZ and YZ views to ensure that the entire ROI was entirely within the area of the bead.

Results

The mean intensity of beads acquired with OptiGrid confocal, OptiGrid wide field, and true wide field imaging are shown in tables 1-3 respectively and plotted in chart 1. Chart 1 shows that of the three techniques used to acquire the data, OptiGrid confocal shows relative intensities closest to the predicted values without any further manipulation of data.

The Hamamatsu Orca ERG camera has a built in DC offset value of about 200 levels. This value is removed from OptiGrid confocal data by the algorithm used to generate the confocal images, but is likely to adversely affect the accuracy of wide field data. We therefore removed 200 gray levels from all wide field mean intensities and 600 gray levels from all OptiGrid wide field mean intensities (OptiGrid wide field images are generated by adding three images). The corrected intensities for OptiGrid wide field and wide field are shown in tables 4 and 5 respectively. Relative intensities for OptiGrid confocal, corrected OptiGrid wide field and corrected wide field are plotted in chart 2. Chart 2 shows all relative intensities closely match the predicted relative intensities following correction of wide field intensities.

Conclusions

1. OptiGrid confocal data is suitable for quantitative analysis of fluorescence intensity.
2. Automatic removal of camera DC offset in the generation of OptiGid confocal images simplifies quantitative analysis compared to wide field techniques.

Bibliography

Neil MAA, Juskaitis R and Wilson T (1997) Method of obtaining optical sectioning by using structured light in a conventional microscope. *Optics Letters* 22: 1905-1907

Puch AC (2005) Resolution Performance in Structured Illumination Microscopy: Comparison of OptiGrid to Traditional Confocal Microscopes. Independent Resolution Testing. Thales Optem technical note.

Table 1. Grid Confocal

Known Relative Intensity (%)	Mean Intensity (SD)	n	Measured Relative Intensity (%)
100	2177 (200.5)	15	100
33	686.7 (81.3)	12	31.5
11	249.5 (17.1)	5	11.4
3.4	82.7 (3.3)	11	3.8
1.2	29.5 (1.3)	9	1.4

Table 2. Grid Wide field

Known Relative Intensity (%)	Mean Intensity (SD)	n	Measured Relative Intensity (%)
100	4664.8 (414.4)	15	100
33	1881.2 (113.7)	12	40
11	1000.6 (25.2)	5	21
3.4	732.1 (6.4)	11	15.7
1.2	634.7 (2)	9	13.6

Table 3. Wide field

Known Relative Intensity (%)	Mean Intensity (SD)	n	Measured Relative Intensity (%)
100	2887.9 (115.9)	4	100
33	1003.2 (31.6)	5	34.7
11	470.4 (6.7)	9	16.3
3.4	284.2 (4.4)	9	9.8
1.2	226.8 (2.7)	9	7.8

Table 4. Grid Wide field Corrected

Known Relative Intensity (%)	Corrected Mean Intensity	n	Measured Relative Intensity (%)
100	4064.8	15	100
33	1281.2	12	31.5
11	400.6	5	9.9
3.4	132.1	11	3.3
1.2	34.7	9	0.9

Table 5. Wide field Corrected

Known Relative Intensity (%)	Corrected Mean Intensity	n	Measured Relative Intensity (%)
100	2687.9	4	100
33	803.2	5	29.9
11	270.4	9	10.1
3.4	84.2	9	3.1
1.2	26.8	9	1

Chart 1. Measured Intensity vs. Known intensity (uncorrected data)

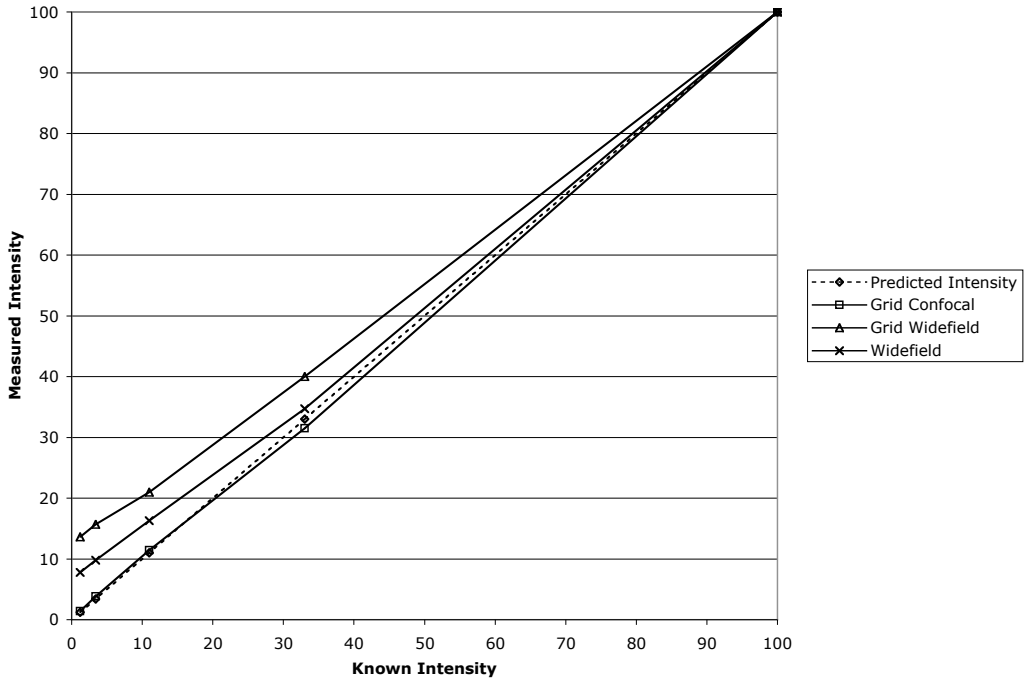
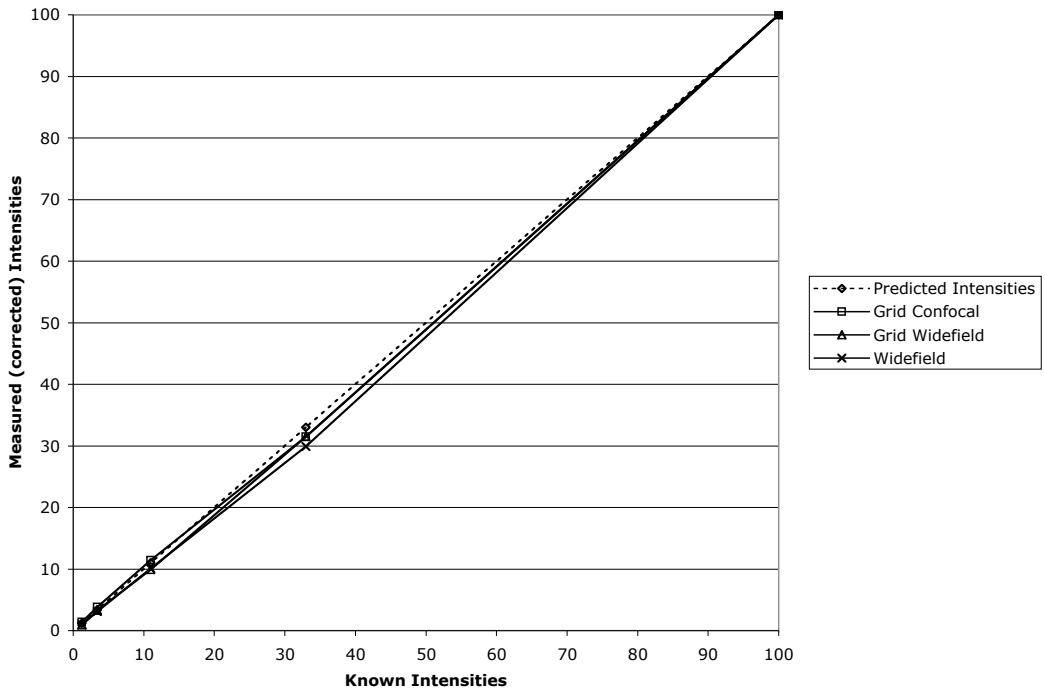


Chart 2. Measured Intensity vs. Known intensity (corrected data)



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