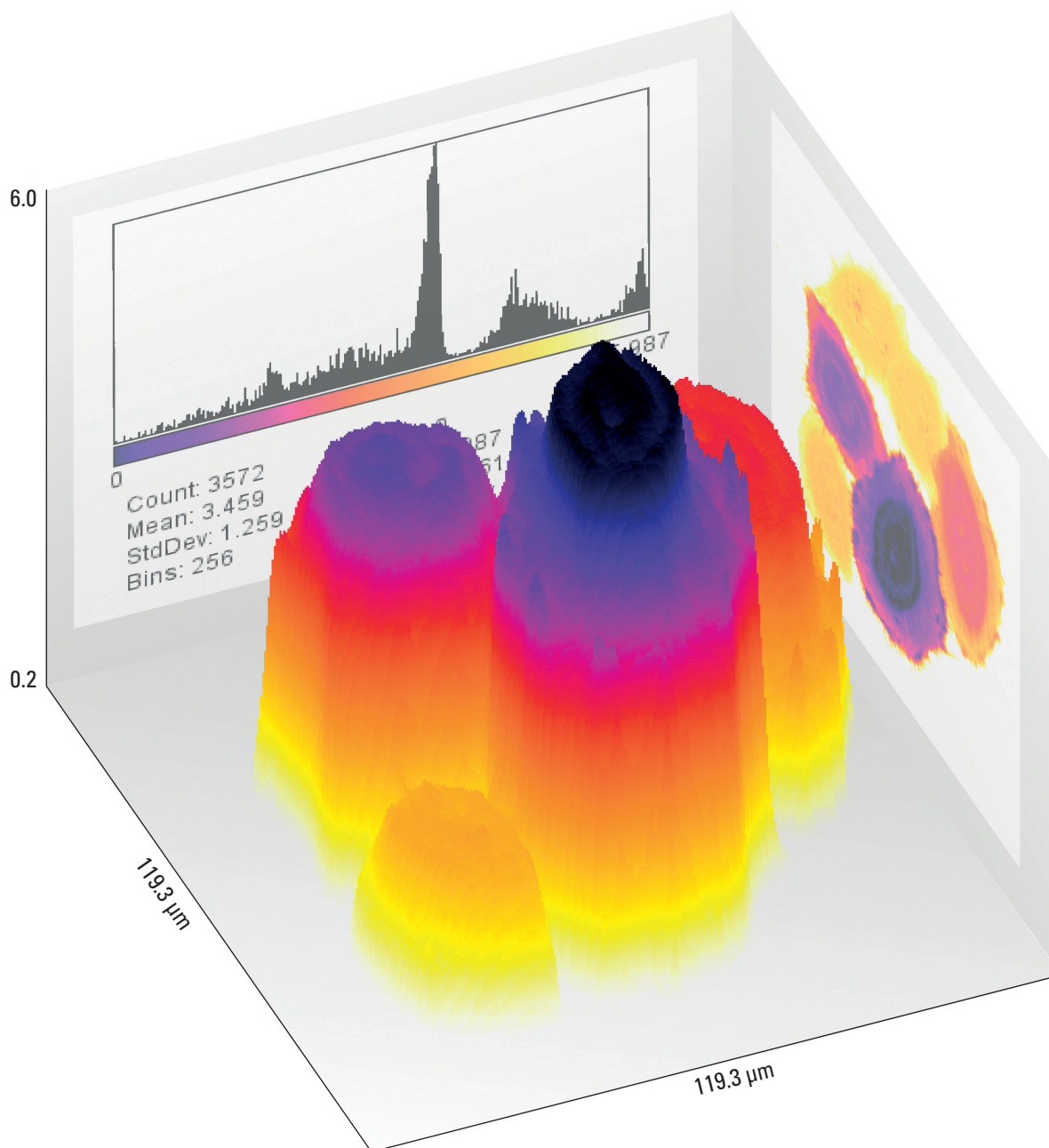


CONFOCAL APPLICATION LETTER

# reSOLUTION

Simple Number and Brightness Analysis with the  
Leica TCS SP5 and HyD Detection



## Introduction

Biochemistry is ruled by dynamic non-equilibria where the concentration of certain biomolecules determines the fates of metabolic or signaling pathways. Not surprisingly, the understanding of labeled biomolecule concentrations *in vivo* allows us to infer biological states and the probability of the fates of single cells. This application letter outlines how to use photon counting in conjunction with HyD detection to measure molecule numbers and their brightness using the N&B approach [1] and ImageJ [2].

### Why N&B with photon counting detectors?

Number and brightness analysis represents a statistical analysis of confocal microscopy images, which yields the mean number of particles  $\langle N \rangle$  and their corresponding brightness  $\langle B \rangle$ . Brightness in this context refers to the number of photon counts per molecule per unit time. As outlined below, relatively straightforward statistics can yield the apparent molecule number and brightness, which are each directly proportional to the absolute molecule number and their emission coefficient. By means of calibration with background, one can even derive these absolute numbers [1]. However, being restricted to the analysis of a single fluorescent species (per detection channel) the method can give insight into aggregation states via their brightness. Both parameters,  $N$  and  $B$ , can be obtained with spatial resolution, thus giving insight into the spatial distribution of biomolecules as well as hints toward the state of mono- or oligomerization. It has been demonstrated that “multi-channel N&B”, termed ccN&B, may reveal molecular interactions. Related techniques on single-point measurements are FCS (Fluorescence Correlation Spectroscopy) and PCH (Photon Counting Histogram) [3,4,5]. As described in [1] N&B requires additional calibration for detector offset when used with detectors operated in integration mode. This additional step is not necessary when using photon counting detectors. In the following application we will use HyD detectors in photon counting mode to generate the images for analysis.

### Statistics behind N&B

The parameter  $B$  studies the statistical fluctuations of fluorescence intensity  $\langle I \rangle$  over time and relative to the total intensity  $\langle I \rangle$ . It can be defined as  $B = \sigma^2 / \langle I \rangle$  (eqn. 1), where  $\sigma$  represents the standard deviation and  $\langle I \rangle$  the total or mean intensity. Thus, if there is no dynamic fluctuation, i.e., the molecules are immobile, the  $B$  parameter converges to 1, otherwise it will be  $> 1$ . The number of molecules is defined as the ratio of total intensity to  $B$ , thus  $N = \langle I \rangle^2 / \sigma^2$  (eqn. 2).

## Practical Realization

### Prerequisites for microscopy

Since the method is based on the inherent timing of a scanning system, the method lends itself directly to confocal microscopes. Samples must have intensity fluctuations due to transport or binding/unbinding. Hence, fixed slides will not work. It is important to choose the imaging conditions such that the pixel dwell time approximates the diffusion time (through the confocal volume) of the labeled particle (see the Application Letter on FCS [5]). The laser power should be low to avoid bleaching. However, enough photons per pixel need to be sampled to achieve appropriate statistics. A good starting point is  $> 20$  photons per pixel. Photon counting detectors are ideal for this kind of analysis, because the usual calibration for offset and  $S$  parameters, as is the case with analog devices or detectors running in integration mode, is not needed here [1]. Therefore, we employ HyDs by default, except where explicitly stated otherwise.

### How to apply number and brightness analysis using ImageJ

While there is specialized software for number and brightness analysis [6], a few simple standard functions in the widely available ImageJ help to get started on the topic. Loading the images into ImageJ can be accomplished either via TIFF images (File/Import/Image Series) or directly from a .lif file using the LOCI importer [7]. We can apply the above mentioned statistics to a time series of confocal images using standard

# Simple Number and Brightness Analysis

stack operations found in the stack menu of ImageJ (Figure 1). Since integer arithmetics can lead to round-off errors that will propagate, in the calculations, the first step after import of an image sequence is to convert it to 32-bit type.

The images (or image maps) we need to create are: mean intensity  $\langle I \rangle$ , variance ( $\sigma^2$ ), squared mean intensity  $\langle I \rangle^2$  as well as N and B.

### Generation of mean intensity map $\langle I \rangle$

This is a straightforward operation called Z-Projection in ImageJ with the default setting “Average Intensity” (Figure 2).

### Generation of variance map $\sigma^2$

The variance is the squared standard deviation. We use the same z projection as above, just using the setting “standard deviation”. Afterward we use “Process/Math/Square” to obtain the variance.

### Generation of squared mean intensity map $\langle I \rangle^2$

This map needed for intermediate calculations is generated from the mean intensity map by copying it (Press Ctrl+C, then “File/New/Internal Clipboard”) and squaring it as above.

### Generation of the B map

According to eqn. 1 we can generate this map by dividing the variance by the mean intensity. We do this in ImageJ using the image calculator found in the “Process” menu (Figure 3). There, we choose – Variance.tif as Image 1, “Divide” as the operation, and “Mean Intensity.tif” as Image 2. We check both options and obtain the B map.

### Generation of the N map

Analogously we use the image calculator to create the N map with the squared mean intensity map as image 1 and the variance as image 2.

Thus, both the N and B maps are obtained. Since executing all steps manually can become quite cumbersome when multiple experiments are analyzed, we can automate these steps with an ImageJ macro. An example of a simple macro is provided in the appendix.

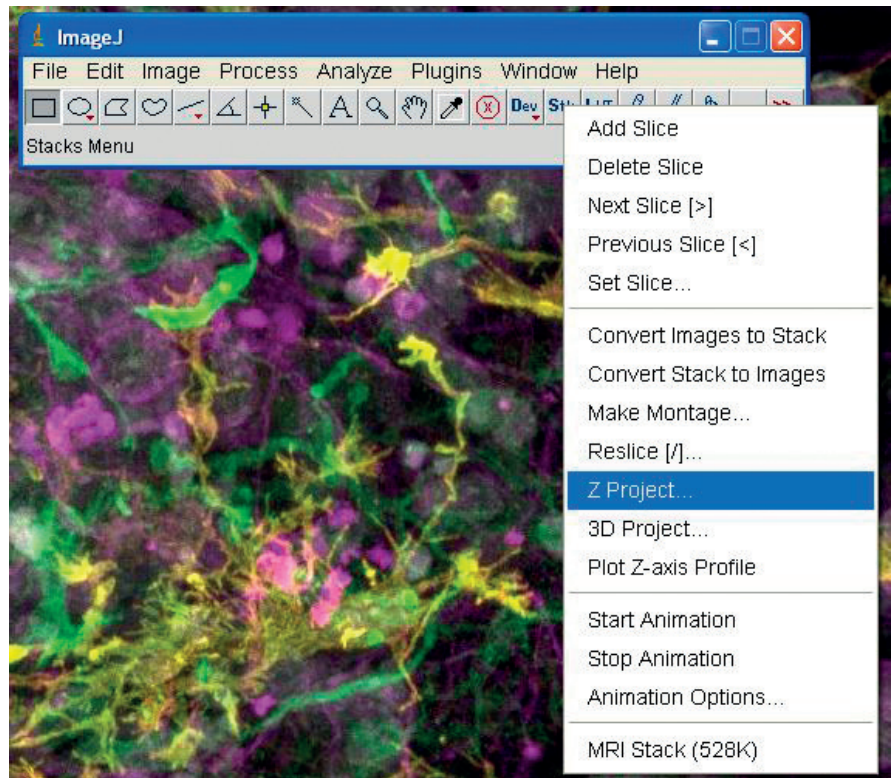


Figure 1: Stacks Menu of ImageJ

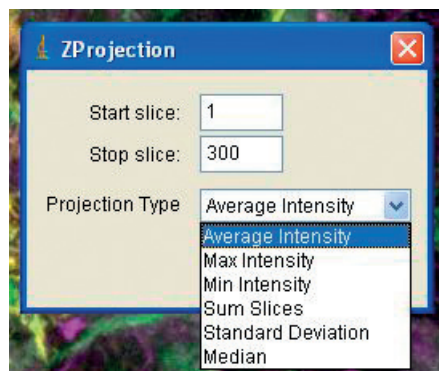


Figure 2: Average Projection

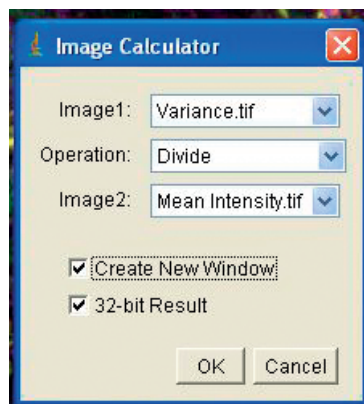


Figure 3: Image Calculation

# N & B Analysis on Fluorescent Beads

A simple tool to test the methodology is to use fluorescent beads. In the example we used 40 nm Y-G beads from molecular probes [8]. The beads were diluted in Millipore water [9] and imaged in a Lab-Tek chamber [10]. Imaging conditions were chosen as listed in Table 1.

Using these settings, 300 images were acquired and loaded into ImageJ. Occasionally, large aggregates of beads are visible in the field of view. These images are deleted so as not to distort the statistics. The results are seen in Figure 4 .

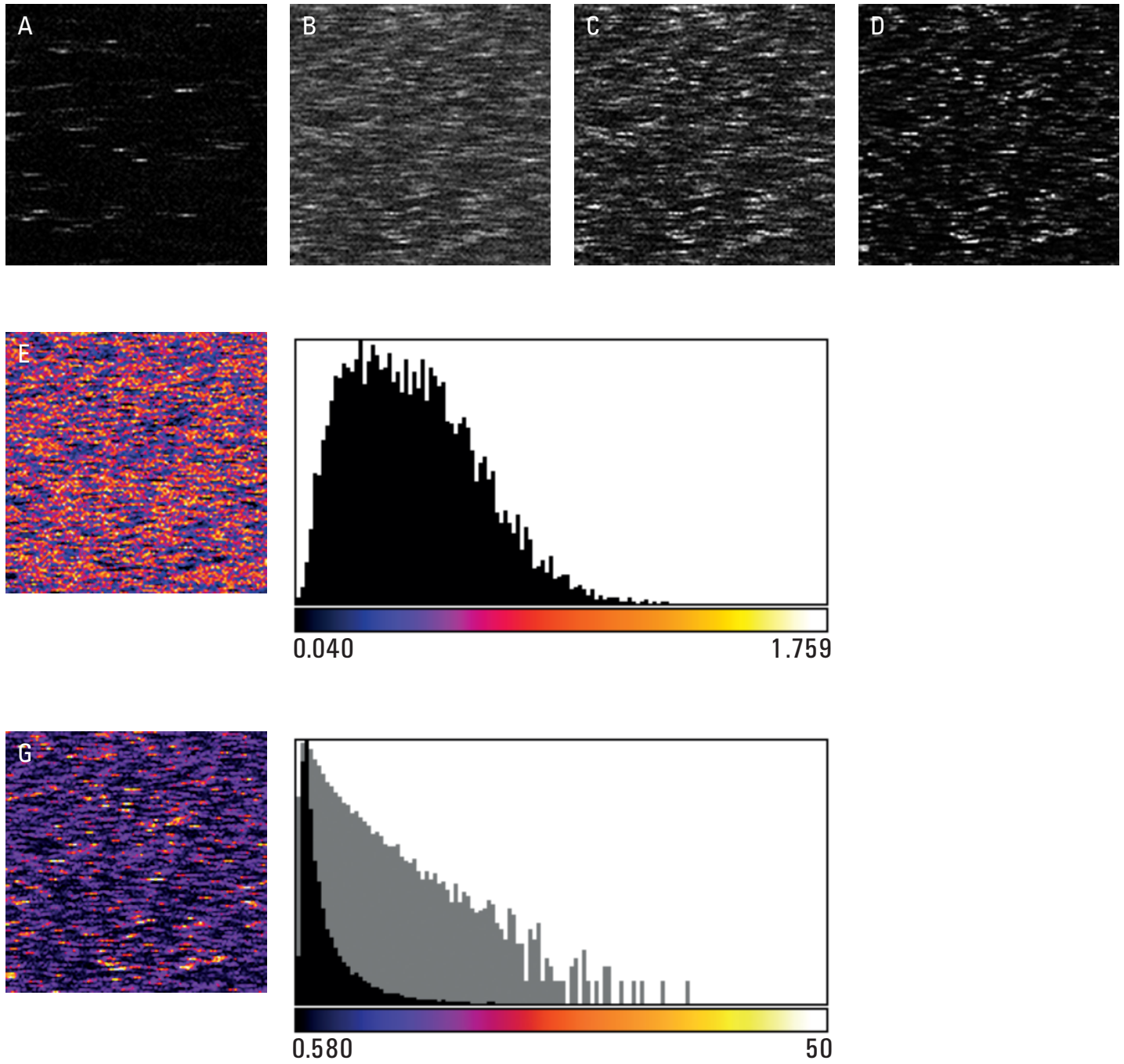
**Table 1**

Scan format	128 x 128
Number of images	300
Line frequency	100 Hz
Zoom	38.7
Voxel size xy	50 nm
Objective	HCX PL APO CS 63.0x1.20 WATER UV
Laser	Argon, 488 nm, tube current 30%, AOTF 2 %
Detector	HyD in photon counting mode

## Comments

In this application letter we have seen how to apply simple analysis of (apparent) number and brightness maps using the Leica TCS SP5 with HyD detection and purely open source tools for image processing. In practice, some caveats apply that need to be accounted for. Photobleaching, omnipresent in fluorescence imaging, leads to a detrending of mean intensities. It can be treated as described in [1]. In our example, photobleaching was below 10% and was not taken into account for the analysis.

# Simple Number and Brightness Analysis



**Figure 4:** N&B analysis on 40 nm Y-G beads excited at 488 nm with an Argon laser. First image of data set (A), mean intensity map (B), squared mean intensity map (C), variance (D). The N map is shown in (E), the corresponding histogram in (F), likewise for the B map and corresponding histogram plotted on linear (black) and log scale (gray) (G, H). The N value peaks at 0.28 and the B value peaks at 1.32.

## References

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2. <http://rsbweb.nih.gov/ij/> (ImageJ)
3. Rigler, R., Widengren, J., "Ultrasensitive detection of single molecules by fluorescence correlation spectroscopy." *Bioscience* 3:180-183 (1990)
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5. Resolution CONFOCAL APPLICATION LETTER No. 36:  
"Fluorescence Correlation Spectroscopy – The femtoliter test tube – system calibration and *in vitro* applications.", Leica Microsystems (2009)
6. <http://www.lfd.uci.edu>
7. <http://loci.wisc.edu/bio-formats/imagej>
8. Molecular Probes FluoSpheres® sulfate microspheres, 0.02 µm, yellow-green fluorescent (F-8845), Life Technologies GmbH, Frankfurter Straße 129B, 64293 Darmstadt, Germany
9. Water GPR Recapur, BDH Prolabo, VWR International GmbH, John-Deere-Str. 5, 76646 Bruchsal
10. Lab-Tek (order no. 155411), Nunc GmbH & Co. KG, Robert-Bosch-Straße 1, D-63505 Langenselbold, Germany

## Appendix

This macro example automates the calculation of both B and N maps in ImageJ v1.4. It assumes the source image stack has been loaded and selected. To run it, copy the information below into a text file and save it into ImageJ's plugins folder (or a subdirectory thereof). Don't forget to restart ImageJ.

```
rename("source_stack");  
  
run("32-bit");  
  
run("Z Project...", "start=1 stop=50 projection=[Average Intensity]");  
  
rename("Mean Intensity");  
  
run("Copy");  
  
run("Internal Clipboard");  
  
run("Square");  
  
rename("Squared Mean Intensity");  
  
selectWindow("source_stack");  
  
run("Z Project...", "start=1 stop=50 projection=[Standard Deviation]");  
  
run("Square");  
  
rename("Variance");  
  
imageCalculator("Divide create 32-bit", "Squared Mean Intensity", "Variance");  
  
rename("N");  
  
imageCalculator("Divide create 32-bit", "Variance", "Mean Intensity");  
  
rename("B");  
  
selectWindow("N");  
  
run("Enhance Contrast", "saturated=0.5");  
  
selectWindow("B");  
  
run("Enhance Contrast", "saturated=0.5");  
  
selectWindow("Squared Mean Intensity");  
  
run("Enhance Contrast", "saturated=0.5");  
  
selectWindow("Variance");  
  
run("Enhance Contrast", "saturated=0.5");
```

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