Workflow to evaluate the Dynamic Range of microscopes and facilitate High Dynamic Range imaging.

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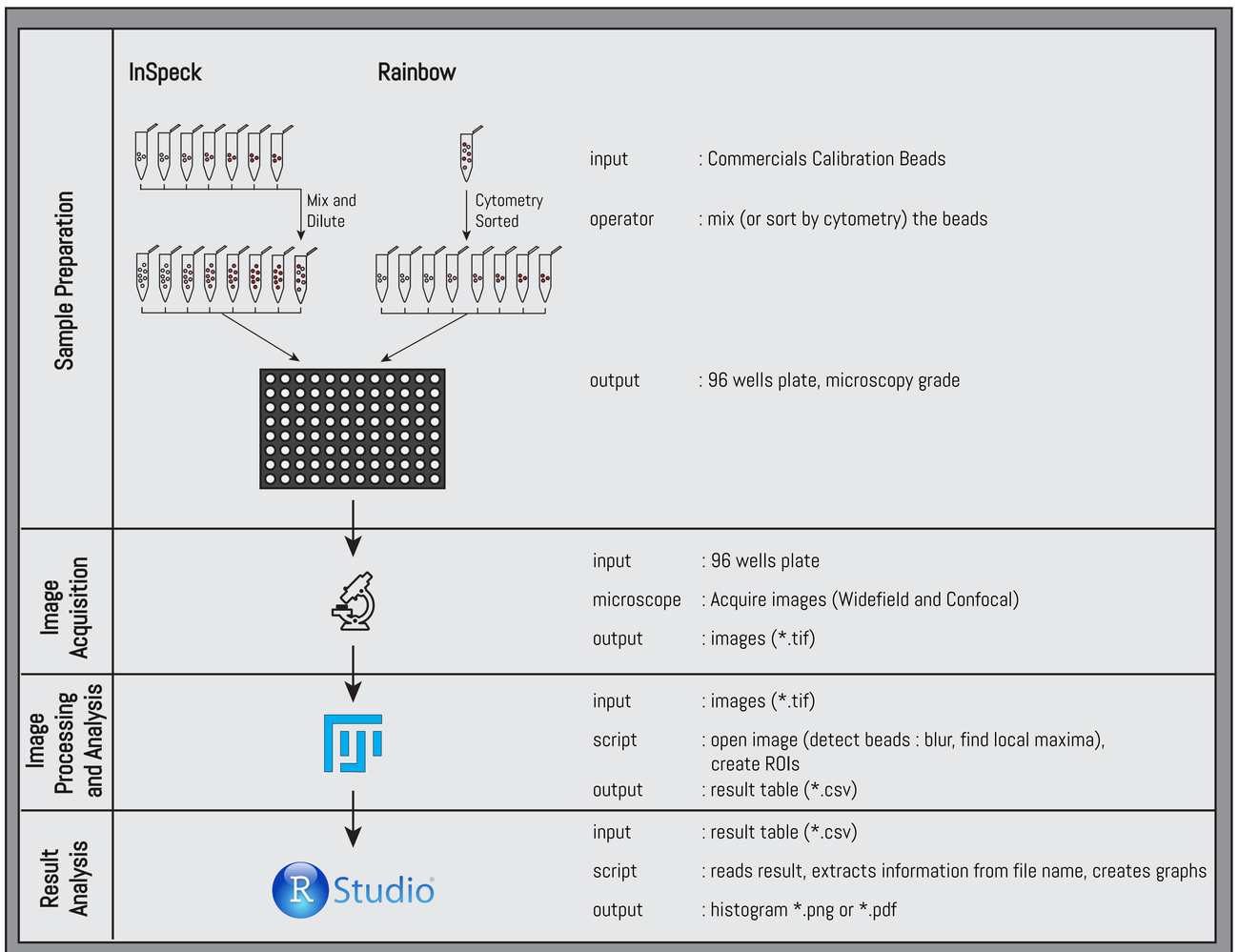
Fluorescent microscopy has turned into an invaluable tool for life sciences within the last 30 years. However due to a variety of reasons quantification of the obtained signals providing a reliable relationship between the analyte (e.g. the protein of interest tagged with a fluorescent marker) and the detected signal is challenging. The concentration of a protein can varies substantially over time and/or locally (different levels in different cellular compartments) leading to a heterogeneity of the acquired signal with a fluorescent microscope over several orders of magnitude.

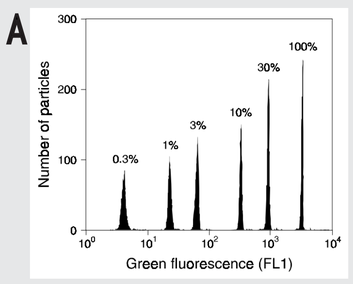
In order to enable quantitative analysis of microscopy images, or render restauration techniques (e.g. deconvolution) more reliable, it is necessary to acquired images with sufficient signal to noise and/or background ratio. Therefore, the ability to detect and distinguish intensity levels over a large intensity range (without saturation of the detector) will rely on the dynamic range capability of the detector as well as of the imaging modality.

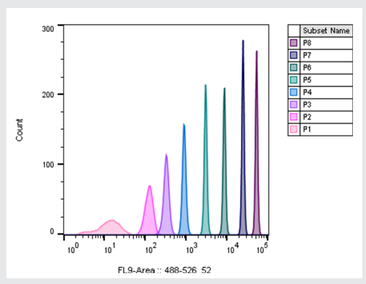
WORKFLOW



Sample Preparation

Rainbow Calibration Particles from Spherotech (RCP-30-5A), also commonly called “8-peak beads” were run and sorted on a Beckman Coulter MoFlo Astrios EQ cell sorter using the 488nm laser (100mW) and the 526/52nm detector. For each of the detected 8 populations, 90,000 beads were sorted using the purity mode into separate wells of a 96-well plate (P1-P8) with a final volume at 200µL. As a control, one mix of beads was added to a different well (P9).





A. InSpeck calibration kit contains 7 vials of beads, 6 with calibrated intensities levels, and 1 without fluorescence. Beads display fluorescence in a narrow spectra (ref I-14787, Molecular Probes)

B. Rainbow calibration kit contains 1 vial, which contains beads of 8 different intensities levels. Beads are visible in a wide fluorescent spectrum. (ref. RCP-30-5A, Spherotech Inc.)

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Image Acquisition

The widefield images were acquired with In Cell Analizer 2200 (GE Healthcare) using the 20x air objective N.A 0.75 . Illumination is performed through a Solid State Illuminator (based on LED technology). Excitation and emission are defined by a combination of fluorescence filters respectively 542/27 and 597/45. Camera is a large field of view sCMOS, 2048x2048 resolution. Images have a dynamic range of 16 bits, exposure times of 0.2 ms.
The confocal images were acquired with a Leica SP8 sysem (inverted Dmi 8 stand), in confocal mode using a HC PL APO 20x/0.75 IMM with oil. Illumination was performed with a White Light Laser. For the 8 peak beads we used a 488nm excitation and a detection range between 498nm and 551nmm, while for the InSpec beads the excitation waveleght was 561nm and the detection range between 571nm and 630nm. Images have a dynamic range of 12 bits. Detection was realized with a HyD detector in photon counting mode with 4 frames accumulation. For the 8 peak beads we used 16 line accumulation, while for the InSpec just 6.
Widefield images wew downsampled to match pixel size of confocal images for comparaison.

Image Processing and Analysis

An ImageJ/Fiji script (using macro language) was used to measure images. The scripts uses a blurred version of the image, looks for local maximas, creates a series of circular ROIs before measuring intensities. The script outputs a csv file.

Result Analysis

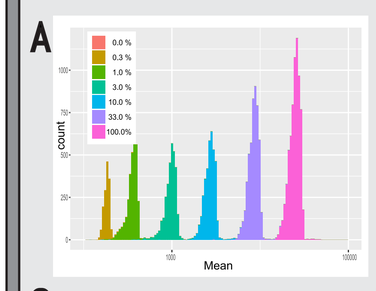
A “R script” was set up and used to generate Distribution graphs.

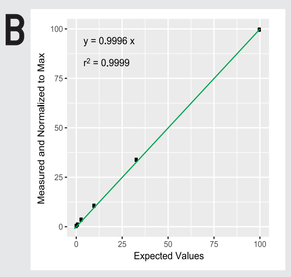
Dynamic Range

Three images of each wells were acquired with a widefield or confocal microscope. The image analysis script allowed to detect beads and to create circular Regions of Interest (ROIs) , that were used to measure for each bead its average intensity.

InSpeck

The distribution of beads intensities are plotted in graphs A and C,respectively for widefield and confocal images.
InSpeck beads are expected to have relative intensities of 100%-30%-10%-3%-1%-0.3%. Beads intensities were normalized using the average value obtained for the brightest population. The Calculated Values are plotted against the expected value in graphs B and D, respectively for widefield and confocal images.

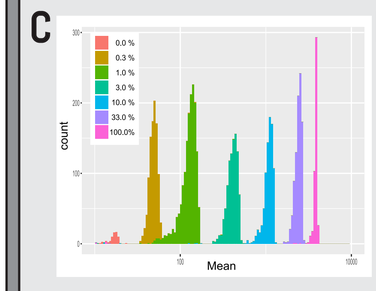


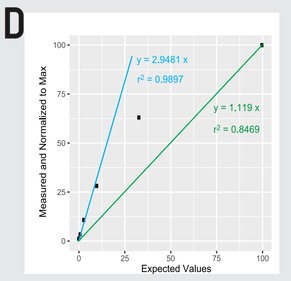


Widefield-16bit

A. 6 peaks can be easily detected. Almost no overlap between the pics

B. Plotting Mean VS Expected value (specification datasheet): linear over the entire range.



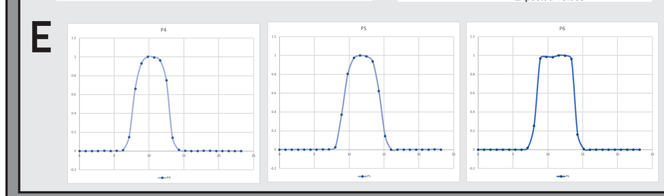


Confocal- 12-bit - Photon Counting

C. 6 peaks can be easily detected. Almost no overlap between the pics

D. Plotting Mean VS Expected value (specification datasheet), linear only on a part of the range.

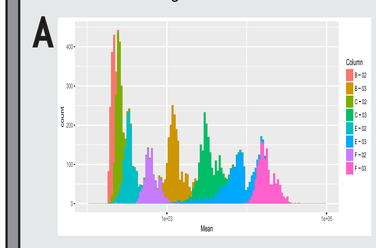
The two highest population are out of linear range.
Either an issue with photon count when high or beads are heterogenous in fluorescence.

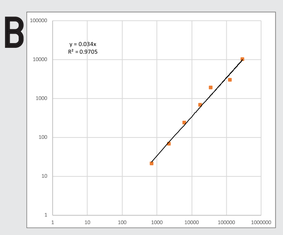


E. Line profile over a bead of each population. Beads are homogenous intensity, but detector is underestimating photon count (even if pixels are not saturated).

Rainbow

The distribution of beads intensities are plotted in graphs A and C, respectively for widefield and confocal images.
The Rainbow beads are expected to have calibrated linear intensities. The average beads intensity of population P1 (deamest one, corresponding to the negative control) was subtracted to the average beads intensity of the other populations. The Calculated Values are plotted against the expected value (Modelcule of Equivalent FITC, from manufacturer datasheet) in graphs B and D, respectively for widefield and confocal images.

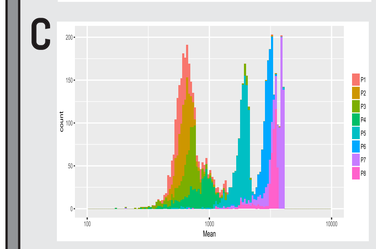


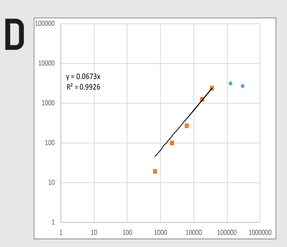


Widefield-16bit

A. 8 peaks can be detected. A bit of overlap between pics. Certainly due to out of focus beads creating artificial variability.

B. Plotting Median (corrected by value of P1) VS Expected value (specification datasheet): linear over all range.

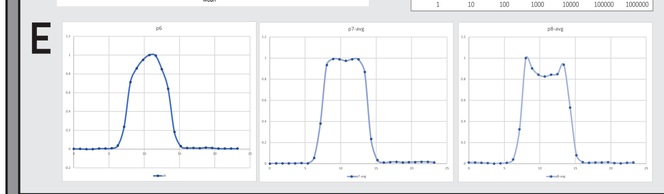




Confocal- 12-bit - Photon Counting

C. 8 peaks can be bearly detected. Large overlap of pics. The Brightest Population of Beads (P8) is second ! Beads look like donut instead of beads

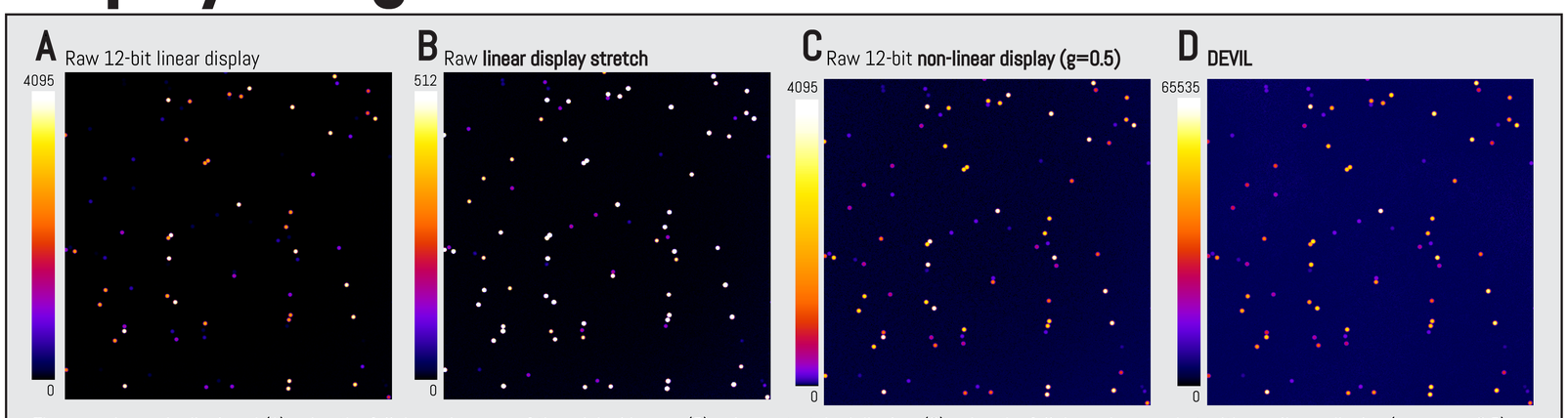
D. Plotting Median VS Expected value (specification datasheet), linear on part of the range.
In confocal



E. Line profile over a bead of each population. Rainow beads are not homogenous in intensity for the brightest population.

Calibration Beads are usefull with Widefields system and 16-bit camera are capable to discriminate up to 6-7 levels of intensities.
Confocal Imaging can reveal heterogenous bead intensities.
Photon counting (with our settings) is not linear over the entire range.

Display Range



The same image is displayed (A) using the full dynamic range of the original image, (B) using a stretched display, (C) using the full dynamic range but with non-linear display (gamma = 0.5) and (D) after appllying DEVIL procedure.
In A., the faintest beads are not visible, while in B. they appear but most of the beads look now saturated and the different intensities level are indistinguishable.
Using a non-linear display (gamma = 0.5) helps to see both faint and bright beads but the different intensities level are still indistinguishable.
Using DEVIL allowed to easily vizualize the different levels each with a different colors.
DEVIL is a workflow that combines several classic Image Processing tools to decrease the difference between high and low-intensity signals within an image. DEVIL is an ImageJ/Fiji script, using the ImageJ macro language. DEVIL uses a combination of three plugins/functions of ImageJ/Fiji: 1) Division by a blurred version of the image, 2) Square Root Calculation, 3) Subtract Background. It was orginally devellopped for lightsheet images, and DEVIL stands for Display Enhancement for Visual Inspection in Lightsheet.

Conclusion

We are presenting a workflow to study and measure the dynamic range of different microscope systems. It is generic and can be applied to microscopes with area detectors as well as to detectors of point scanning confocal microscopes. By using the workflow we were able to conclude that:

- Photon counting detectors have a limited linear range (object brightness real vs. measured).
- 2D images of beads acquired with a confocal microscope lead to a considerable bias when analyzing the brightness

The workflow can be used to for instrument testing, compare different instruments, ensure quantitative imaging as well as to provide an easy approach for High Dynamic Range (HDR) imaging [1].

Bio Imaging & Optics Platform