Background-Based Illumination Bias Correction

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OVERVIEW

In microscopy, both the amount of excitation and emission light that is directed through a microscope objective is spatially non-uniform, and this is referred to as 'illumination bias' (Fig. 1). Accounting for illumination bias is critical for inferring relative expression levels and can be helpful for solving segmentation problems.

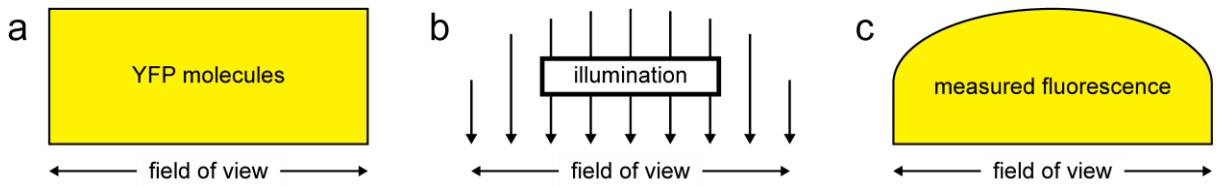

Figure 1. Illustration of illumination bias. (a) Hypothetical uniform distribution of YFP molecules across one dimension of a field of view. (b) Hypothetical distribution of transmission of light across the same field of view. (c) The resulting fluorescence measured by the microscope across the field of view.

There are a number of methods available to try to account for this effect, and here we introduce a method we will refer to as 'background-based' illumination bias correction. This method exploits the assumption that the autofluorescence in a solution (e.g. growth media) is uniformly distributed across a field of view. As illustrated in Figure 2, the technique involves the following steps for correcting a nuclear marker image such as Hoechst or H2B:

1. Generate a dilated mask of the foreground signal.
2. Divide the image into tiles.
3. For each tile, determine the median intensity of only the background pixels (i.e. ignore any pixels within the mask generated in step 1). Repeat steps 1-3 for several images and, for each tile position, calculate the median value across all images for that tile.
4. Interpolate the tile values to estimate a smooth illumination bias.

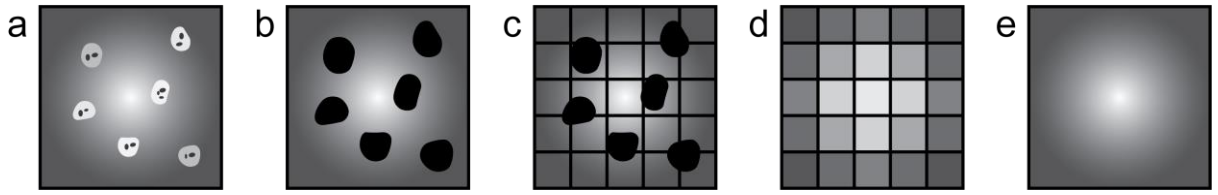

Figure 2. Illustration of background-based illumination bias calculation for a nuclear marker (e.g. Hoechst or H2B). (a) Raw image minus CMOS offset. (b) Generate a dilated mask of the foreground signal to remove from further calculations. (c) Divide the image into tiles. (d) Calculate the median background intensity within each tile. (e) Interpolate the tiles to generate a smoothened approximation of the illumination bias.

It is important to keep in mind that cameras can apply fixed offsets unaffected by the light (Fig.3). To account for this, we simply have to subtract off the offset prior to calculating the bias. The offset can be determined by taking an image with the lamp turned off.

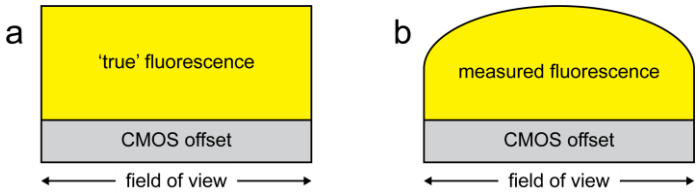

Figure 3. The offset from a CMOS camera is unaffected by light.

The assumption of a uniform background fluorescence also enables us to empirically confirm whether or not the illumination bias has been corrected for a given image, since the corrected background fluorescence should appear uniform across the image (Fig. 4). Since the bias was calculated over many images, a proper correction implies that the illumination bias is common across these images.

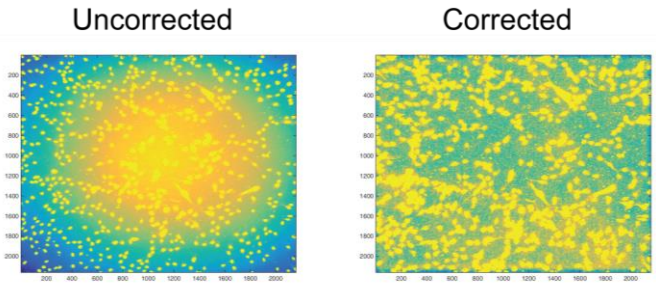

Figure 4. Heat map of DAPI image before and after correction

This raises the question of whether the illumination bias in one image is common to any other image. This likely has to be determined for each microscope. For Molecular Devices IX Micro, the bias is independent of well position within a plate, but is dependent on the site position within a well, and is also dependent on the filter cube used. Thus, an individual bias has to be calculated for each combination of filter cube and site position.

INSTRUCTIONS

1. Obtain the CMOS offset:

   a. Take several (~10) images without any light. This can be done by keeping the lamp off or keeping the shutter from opening.
   b. Run *CMOSOffsetCalc.m*. This will obtain the average intensity of every pixel across the multiple images obtained, and will average out any random fluctuations across the CMOS. This only has to be done once, unless you suspect the CMOS offset has changed (you would notice a slight pattern after illumination bias correction). Thus, save the file *cmosoffset.mat* somewhere to access each time you perform this correction.

2. Calculate the illumination bias:

   a. Take experiment images with 'Shading Correction' option turned off. For each filter channel, set the lamp strength and exposure time such that the background illumination in the dimmest areas is at least 200 RFUs.
   b. Run *BiasCalc_Nuc.m* on the nuclear marker (i.e. H2B or Hoechst). Use all images from imaging session for fixed-cell, and optionally use all wells and multiple frames for live-cell. The script will calculate the bias for each site within a well (i.e. 2x2 sites per well will result in 4 bias calculations). Try to run this with the highest number of tiles possible (31 is the highest necessary), but with fewer or highly confluent images it becomes necessary to reduce the tile number. For at least one site, determine the optimal tile number by visually confirming that the

calculated bias is smooth. For demonstration purposes, I've inserted *imshow()* commands in the script, but these are normally left out.

c. Run *BiasCalc_Signal.m* on the signals (even if it is a nuclear signal, like pRb). The only difference from *BiasCalc_Nuc.m* is that you specify how much to dilate the nuclear mask when trying to sample just the background. I generally do one times the nuclear radius for nuclear signals and two times the nuclear radius for cytoplasmic signals.

3. Correct the illumination bias in each image:

a. Run *BiasCorrection.m* on a sample experiment image. This script just demonstrates that we can indeed correct an image, which simply involves loading an image, subtracting the CMOS offset, and then dividing by the bias calculation. The script details how to view the image, before and after correction, using the *imtool()* function. This enables us to interactively change the saturation limits in order to see the non-uniformity of the background. For illustration purposes, I've determined suitable limits ahead of time and saved a heat map version of the images, which I showed in the Overview section above. I've included an updated version of *Immunostain.m*, which performs bias correction.