IncuCyte™ Background Fluorescence Technical Note

Media Fluorescence

The IncuCyte™ FLR and ZOOM are uniquely designed to enable automated long-term, live-cell imaging of cells as they grow – an imaging strategy that we refer to as Live Content Imaging. This powerful strategy enables researchers to perform long-term, live-cell imaging experiments that last from hours to days in cultures maintained in cell culture media as opposed to a simple buffer solution. Most media formulations contain riboflavin, a media component that is excited and emits fluorescence in the same spectral range as GFP (Figure 1). As a consequence, riboflavin can provide a significant background signal when imaging cells in cell culture media. This technical note discusses the fluorescent properties of riboflavin in cell culture media, the impact that this can have in images acquired with the IncuCyte imaging system and describes an integrated image processing technique that has been implemented into the basic IncuCyte ZOOM software package to minimize the contribution of background fluorescence to analyzed data.

Contribution of Riboflavin to Fluorescence

Riboflavin, an essential vitamin, appears to be the highest contributing fluorescent component when the IncuCyte is configured to excite and read GFP. To determine the contribution of riboflavin fluorescence on images, the fluorescence intensities of seven common media formulations and three concentrations of riboflavin were quantified (Table 1). Each well of a 24-well microplate containing 1 mL of solution was imaged in an IncuCyte™ FLR. The resulting fluorescence values were background corrected by subtracting the fluorescence from control wells containing only deionized water to remove contributions from the plate alone. These results indicate that the majority of green media fluorescence is proportional to riboflavin content when riboflavin is >0.04 mg/L. Below this level, other media components appear to also contribute to low level background. The results in Table 1 were not significantly changed by additives such as fetal bovine serum (FBS), penicillin, or streptomycin. Lastly, contributions with or without phenol red were not specifically tested, however media with phenol red and a given concentration of riboflavin was no more fluorescent than the same concentration of riboflavin mixed with deionized water.

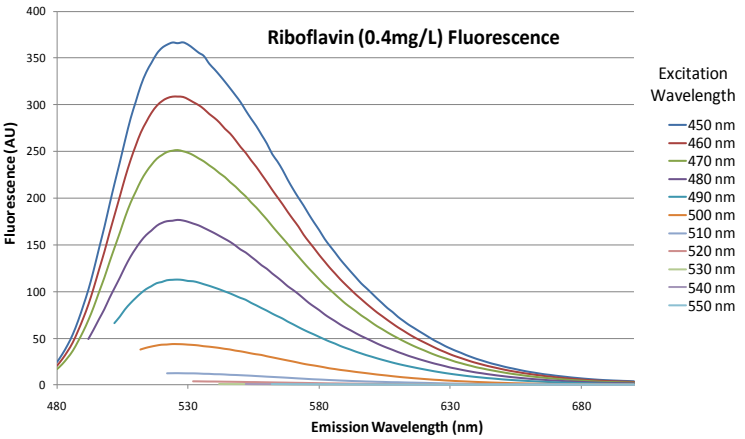

Figure 1: Spectral properties of riboflavin. Excitation of riboflavin between 450 to 490 nm results in emission with a peak at 530 nm.

Spatial Uniformity of the Background

As part of the IncuCyte calibration procedure, which uses a standard slide and fluorescent dye (see IncuCyte™ FLR Calibration Product Note), an image profile is recorded from which spatial differences in image intensity can be corrected. This process is called flat-field calibration and helps to insure that the system has the same relative sensitivity to all objects in the image plane regardless of spatial position. However, several properties of the fluorescence produced by the media background can introduce spatial patterns in the images (Figure 2). These include variation in the intensity of the background across the image (spatial uniformity) as the light propagates up through the well, temporal variation in the background due

primarily to bleaching, as well as changes which are related to the amount of media in the well. In some cases (dim cells, high background), a halo structure will occur (see Figure 2A) with a 10x and 4x objective, and a cross structure will occur (see Figure 2B) with a 20x objective. Note, the halo and cross are a result of actual media background fluorescence and are not an error in the previously discussed flat-field correction which is done for objects in the cell plane.

Media	Riboflavin Concentration (mg/L)	Fluorescence Intensity (Arbitrary Units)
DMEM	0.4	43.6
RPMI-1640	0.2	29.6
DMEM/F-12K	0.2	31.7
FBM	0.11	16.1
Eagles MEM	0.1	12.9
F-12K	0.04	5.4
EBM	0.004	3.7
Riboflavin	0.4	58.7
Riboflavin	0.2	26.5
Riboflavin	0.1	13.0

Table 1: Different cell culture media formulations and concentrations of riboflavin were evaluated for their fluorescent intensity when imaged in an IncuCyte FLR. The Arbitrary Unit (AU) values shown indicate the corrected (plate fluorescence subtracted), average image brightness for each solution tested. AU values indicate the brightness of fluorescent pixels and are relative to the fluorescent standard used to calibrate the instrument i.e. a pixel with a value of 50 AU is 50% as bright as the calibration standard.

At the cellular plane of focus the illumination is at its highest intensity. Laterally, illumination is the brightest in the center and becomes weaker at the edges of the image. However, since the flat-field correction is done in the cell plane, fluorescence originating from levels higher up in the media may be over corrected at the edges resulting in the halo appearance. When imaged with a 20x objective, fluorescence originating from levels higher up in the media may be over corrected in the center resulting in the cross appearance. When using the auto-scaling features of IncuCyte software, the degree to which the halo or cross is visible depends on the strength of cellular fluorescence in the field of view relative to the background. It is not uncommon to have cells that are much brighter than background as is shown in Figure 2C. In this case, the halo structure is almost unnoticeable. Figure 2A and 2B show the opposite case, where cells are only slightly brighter than the media and a halo or cross are observed. In Figure 2, all three images are displayed using the auto-scale feature of the IncuCyte software.

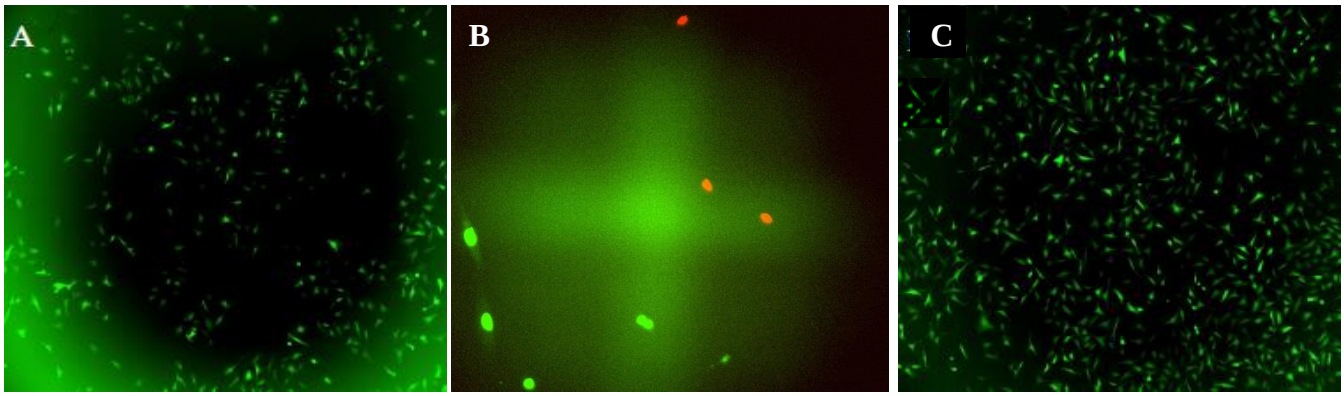

Figure 2: Background fluorescence in media with high levels of riboflavin. (A) A population of relatively weak fluorescing cells in DMEM, a media with a relatively high background fluorescence. Images of cells cultured in DMEM may show a halo effect when acquired with the IncuCyte FLR at 10x or in the IncuCyte ZOOM at 10x or 4x. (B) Imaging GFP and RFP (mkate2) labeled cells in DMEM results in a “cross” effect when acquired with the IncuCyte ZOOM at 20x. (C) Bright cells, low background. Halo is not observed since the cells are significantly brighter than the background.

Background Photobleaching

Riboflavin photobleaches with exposure to light, which results in a reduction in background fluorescence following repeated exposures over time as illustrated in Figure 3A. In this experiment, wells containing 3 different types of media were repeatedly exposed to the blue LED contained within the IncuCyte system over the course of 2.5 minutes. Initial fluorescence intensities correlate to the amount of riboflavin present in the media (refer to Table 1). As the media is repeatedly exposed to the light, the amount of fluorescence decreases as the riboflavin is photobleached. Since the IncuCyte does not expose the whole well to light (in this case, only 9 images per well were acquired in a 24-well plate), non-photobleached riboflavin can diffuse into an image region from surrounding unexposed portions of a well or vessel. Therefore, the rate and extent of bleaching is dependent on the culture vessel size and the number of images per well/vessel. Figure 3B illustrates the relative fluorescence changes for DMEM and GFP. The total decrease in fluorescence values associated with GFP over the same 2.5 hour time course was 8% while the decrease in background fluorescence associated with the media was over 85%. Each data point was normalized using the first value. Taken together, these data establish that GFP is stable to the imaging conditions in the IncuCyte Live Content Imaging system.

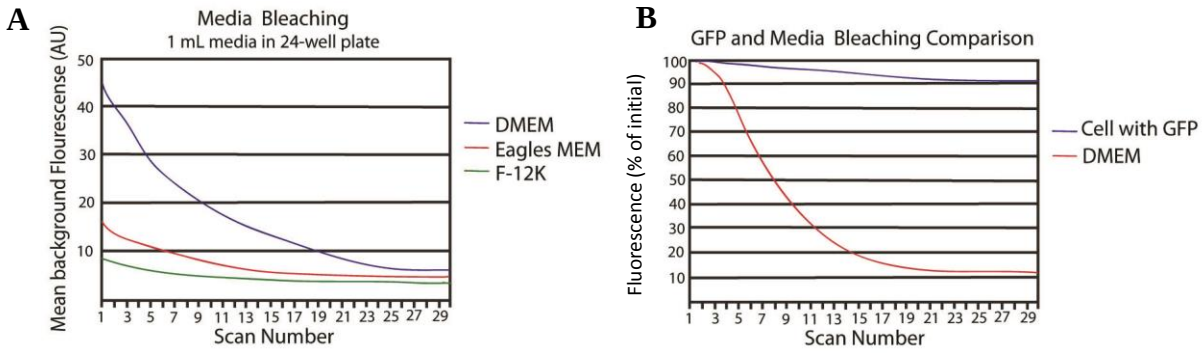

Figure 3: Riboflavin photobleaches faster than GFP. (A) 1 mL of the indicated media was dispensed into one well of a 24-well plate. Wells were imaged 30 times over a 2.5 hour period at 9 images per well. (B) Cells expressing GFP were imaged over an identical 2.5 hour period. Cells expressing GFP were segmented and fluorescence intensities of the GFP expressing cells were obtained in order to measure the amount of GFP photobleaching in comparison to riboflavin photobleaching observed in the culture medium. Fluorescence intensities at each time point were normalized to the initial fluorescence intensity at t=0. Data presented represent the mean intensities found in all 9 images.

The Effect of Media Depth

The IncuCyte is not a confocal imaging system. Therefore, the IncuCyte has significant sensitivity to light emanating from planes well above the focus. Figure 4 shows the average signal in a 24-well plate for various volumes of DMEM media. Because background signal increases with depth, the background levels will vary within a well due to the fluid meniscus. Therefore, to control background levels it is advisable to minimize the media volumes used when working with the IncuCyte.

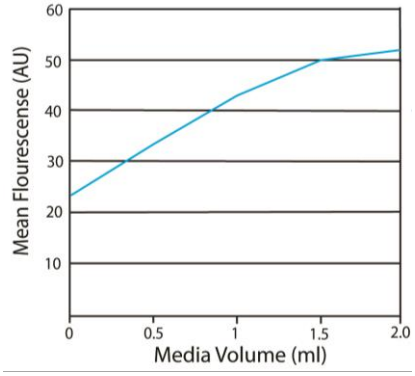

Figure 4: Media depth affects background fluorescence. Increasing volumes of DMEM (high riboflavin) were dispensed into multiple wells in a 24-well plate. Increasing media volumes resulted in increased mean fluorescence. Data represent the mean fluorescence of 9 images acquired per well.

Correction of Background Fluorescence Using Image Processing

Digital image analysis is important for proper identification of fluorescent objects within an image. The basic software package for the IncuCyte contains algorithms to accommodate the non-uniformity of the background within images. These algorithms are discussed in detail below.

Adaptive Segmentation

In contrast to Fixed Threshold segmentation, the Adaptive Segmentation algorithm is used to automatically track background variation across individual images. In doing so, Adaptive Segmentation provides a simple way for the user to input parameters to successfully segment an image with a non-uniform background (Figure 5). In the IncuCyte ZOOM processing definition editor software, users input a threshold above the local background level (LBL). In the IncuCyte FLR software, users provide an estimation of the background and foreground around a dim object. In both cases, the robust algorithm used to segment the image is designed to differentiate fluorescent objects from the background and properly mask them. To accomplish this, the Adaptive Segmentation algorithm first breaks the image into small regions and determines the LBL across each segment. The threshold parameter(s) defined by the user are then used to segment the image (see IncuCyte™ ZOOM Fluorescence Processing or IncuCyte FLR Object Counting Technical Notes for more detailed information). Adaptive Segmentation is a robust and versatile processing method and is a good choice for processing challenging images for which an object count, object confluence, and object shape are of interest. However, it is important to note that the Adaptive Segmentation algorithm estimates the LBL in order to properly segment the image, but **does not subtract local background values when computing object intensities** and does not provide a background subtracted image to the viewer for viewing or export.

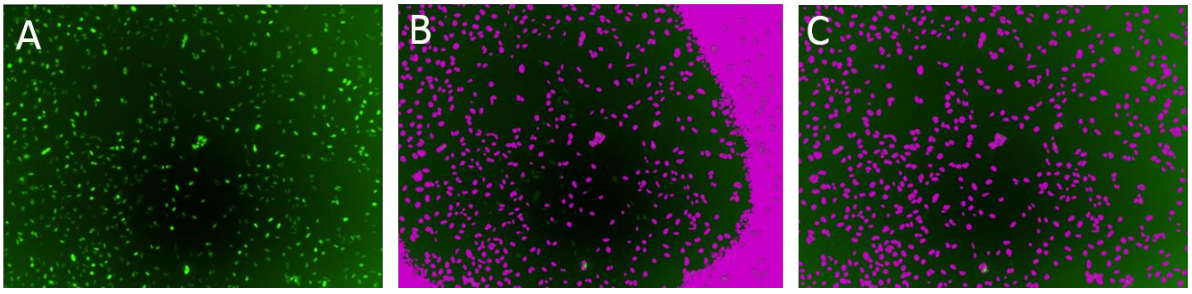

Figure 5: Fixed Threshold vs. Adaptive Segmentation. (A) Original image with high background (imaged in DMEM media) and dim objects. (B) Same image analyzed with Fixed Threshold algorithm. With uneven background, the algorithm fails to mask dimmer cells in the middle and separate the background from the cells on the edges. (C) Adaptive Segmentation algorithm successfully identifies most of the positive objects in these challenging conditions.

Top-Hat Background Subtraction

In order to effectively segment objects in images with highly non-uniform background, as well as eliminate the effect of local background values on object intensity metrics, an additional algorithm is available in the basic IncuCyte ZOOM software package. Top-Hat Background Subtraction This allows researchers to compare the fluorescence intensities of objects within a single image as well as across multiple images.

Top-Hat filtering is a commonly used method by image processors to correct background non-uniformity. In the basic IncuCyte ZOOM software, the user initially defines a disk based on a radius parameter (µm). This value can be determined by choosing the largest fluorescent object and measuring the radius of the object to the background using the IncuCyte measuring tool (Figure 6A). In the case of non-circular objects, the shortest distance from the center of the object to the background should be chosen. If the radius value chosen is significantly too large, the algorithm may lose the subtle background gradient changes. If the radius is set too small, partial loss of objects may be observed. In general, it is better to overestimate the radius by at least 30%. Once the disk is generated, it moves systematically through the image comparing each pixel's neighbors within the disk to determine the local background (Figure 6B). The local background is then subtracted from the original image resulting in a background subtracted image (Figure 6C). A threshold value is then set by the user to properly segment the image. Following this method of segmentation, the fluorescent properties of the objects do not include the background component and can be used for comparative analysis (Figure 7). Furthermore, the resulting background subtracted images are available to export for movies, image sets, and presentations.

For a step-by-step guide on how to apply Adaptive Segmentation or Top-Hat background subtraction within the processing definition editor, please refer to the Fluorescence Processing Technical Note.

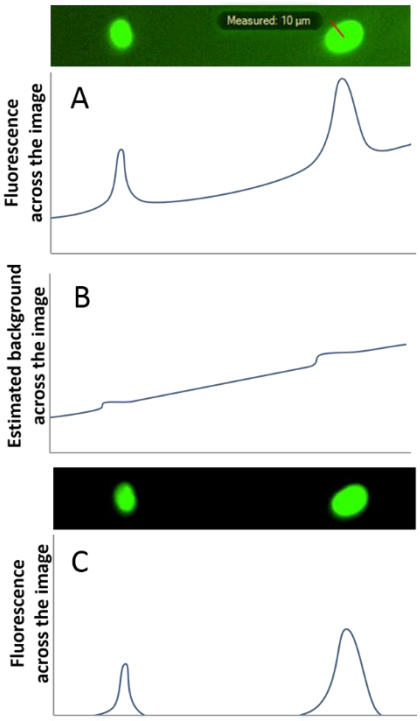

Figure 6: Top-Hat Background Subtraction Algorithm. (A) Original image. The line graph indicates the spatial fluorescence intensities across the image. (B) The filtering algorithm estimates the background across the image using a disk of user defined size. (C) The estimated background is subtracted from the original values resulting in a background corrected image.

Conclusions

When it is possible, we recommend using low-fluorescence media, i.e. media with low riboflavin to avoid background non-uniformity (Table 1). If this option is not available, both Adaptive Segmentation and Top-Hat Background Subtraction algorithms are designed to handle non-uniform background effects. Using the Top-Hat Background Subtraction method has the advantage of producing raw fluorescence intensity values and generating unambiguous images.

Figure 7: Example of Object Properties Following Top-Hat Background Subtraction. (Left) Example of an image with non-uniform background fluorescence. (Right) Same image, after processing with Top-Hat background subtraction. Note that with the uneven background, the object on the left appears to be dimmer than the object on the right. However, after Top-Hat filtering has been performed, the computed object intensities indicate that the object on the left is slightly brighter than the object on the right.