

Absolute Positioning Control: Applications in Imaging, Pharmacological Delivery, and Functional Genomics with Live Cells

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Summary

Successful high-resolution fluorescent imaging of live cells requires overcoming a number of technical hurdles. These hurdles include uniform delivery of low-density light and the accompanying need for highly sensitive detection; mathematical restoration of blurred light; and precision placement and re-placement of the cell within the field of view of the microscopy system. Applied Precision, LLC has successfully exercised its experience in precision motion control to clear the last of these hurdles. Precision motion is a fundamental requirement for accurate 3-dimensional data acquisition as it ensures that all three axes (X, Y, and Z) can be moved independently both with great accuracy and with no cross-coupling between the axes. The same precise XYZ

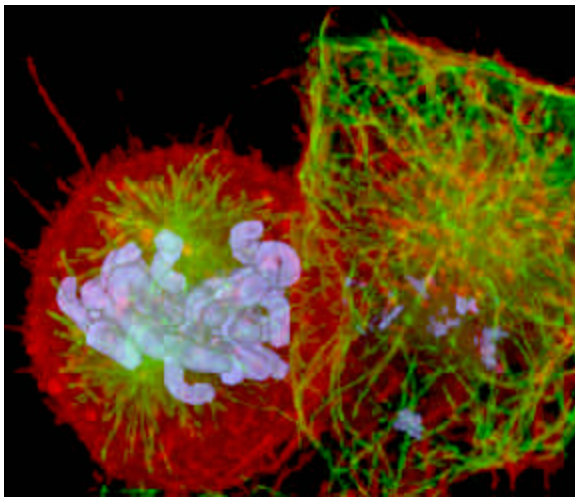


Figure 1:
Fixed CHO cells stained for tubulin (green), actin (red), and DNA (blue-grey).

stage can be used to move and re-position the sample within the field of view, which enables accurate imaging of multiple cells in a time-course study. This "point-visiting" capability enables many cells to be studied within the same experiment thus increasing the efficiency of each study. It also opens the door to applications such as integrating micro-injection systems with point visiting to automate micro-injection and enable time-course studies of genetically, biochemically, and biomechanically manipulated cells.

Live-cell Imaging

Long-term, high-resolution studies of living cells represent one of the more interesting engineering challenges in biotechnology. Xe, et al. [1], Marshall et al. [2], Danuser et al. [3], and many others have demonstrated the utility of high-resolution imaging to study sub-cellular pharmacological effects. In these studies, they have shown that profound pharmacological effects can be initiated by subtle changes in the three-dimensional morphology of sub-cellular structures. For example, Xe in Sorger's lab at MIT has demonstrated that pharmacologically induced changes in the kinetichore are associated with check-point initiation [4].

While generating sub-cellular, three-dimensional images of fixed cells is fairly routine (Figure 1); working with living cells is much more challenging. Many laboratories have found that the best way to scan live cells is by means of a fully integrated Image Restoration Microscopy System such as the DeltaVision RT system developed by Applied Precision, LLC. This system delivers diffraction-limited lateral and axial resolution even while imaging living cells over relatively long time-courses. This Image Restoration Microscopy system, used in conjunction with auto-fluorescent proteins (AFP's) such as GFP variants and DsRed, has enabled laboratories to follow multiple probes in living cells with high spatial and temporal resolution.

Absolute Positioning Control

The DeltaVision system is able to achieve this by a carefully integrated design of mechanics, electronics, optics, and software specifically targeted for this application. The light path, excitation light intensity, fluorescence detection, and positioning must all be tightly controlled for optimal performance. Some vendors have attempted Image Restoration Microscopy, but by failing to control all of these parameters the resolution on these systems suffer. In the DeltaVision system, all microscopy parameters have been engineered to eliminate and/or track the systematic changes that can occur while an experiment is running.

The DeltaVision Image Restoration Microscopy System uses iterative deconvolution methods to extend the resolution of the fluorescence microscope. In order to achieve this, data registration is paramount. There are at least two different forms of registration that must be accounted for. On one hand, the data must be registered in terms of intensity. Since the emission intensity of the fluorescence signal is proportional (over some range of intensities) to the excitation intensity, the excitation intensity must be corrected for and controlled. The other form of registration is spatial. All of the pixels in the stack of images must line up with each other. Movements in one dimension must not introduce movements in the other spatial axes. For example, Z (or axial) movements must not introduce movements in either lateral axes (X or Y) (i.e., no cross-coupling between axes). In addition, the movements in all axes must be both accurate (a movement of a particular size must be correct) and repeatable (a movement of a particular size must be the same every time). The DeltaVision system performs iterative deconvolution in true spatial coordinates so that if the steps between sections are described as being a particular interval (say 200 nm) then they must be correct. In time-lapse experiments, it is important that all of the stage movements move the point of imaging to the to the same place every time, that is, the repeatability must be good as well. These factors, taken together, define absolute positioning (Figure 2).

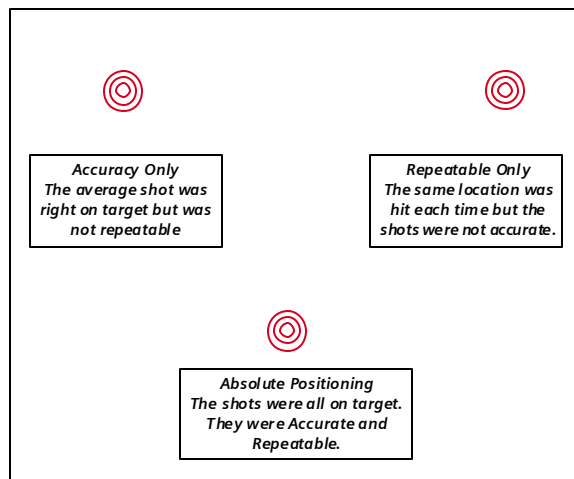


Figure 2

Absolute Positioning in Live-cell Imaging

In many cellular model systems, there is no homogenous cell population. Cells vary based on cell phenotype, relative transcription levels, transfection efficiencies, cell cycle, etc. Consequently, many cell biology experiments are inefficient because the instrumentation limits the user to a single visual field of cells. If the desired event does not happen in the observed cell, the entire experimental observation may be wasted. In long timecourse experiments, this can be very costly. The absolute positioning characteristics described above (low cross-coupling, high accuracy, high repeatability), if carried over sufficient lateral travel, make it possible to follow many cells within the same experiment. In this model, the user marks the locations of several cells of interest. At each time-point, the system will acquire a three-dimensional data set at each location marked (Figure 3). If any of the marked cells undergo the desired event, then the experiment will yield the needed data. By trading off temporal resolution and cell homogeneity (i.e., phenotypic and genotypic uniformity) the user can create conditions under which virtually every experiment yields useful data.

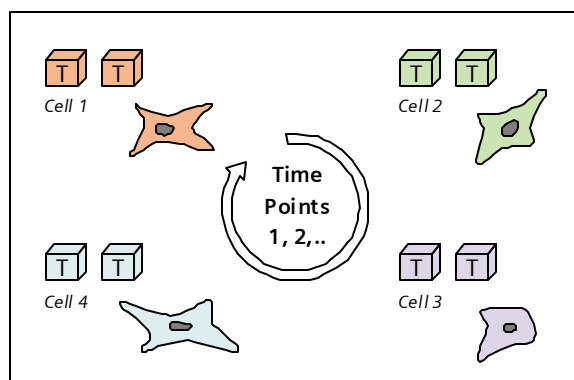


Figure 3

Absolute Positioning Combined with Micro-Injection

In another type of experiment, cell biologists would like to perturb specific cells while documenting cellular processes. Micro-injection is a well-established methodology for introducing these perturbations. This methodology has been used to mechanically stimulate cells, to deliver pharmacological agents and agonists, to deliver gene expression vectors to the nuclei of cells, to deliver proteins to the cytoplasm, to deliver specific mRNA's, to introduce anti-sense mRNA's to knock out gene function, and a host of other applications.

One of the difficulties in this class of experiment is that many cells do not tolerate these manipulations. Cell morbidity and mechanical destruction of the cell are common. In some cell types, it is common for the manipulation of the cell to initiate apoptosis. As a result, these experiments are less efficient than the experiments described in the previous section.

With these difficulties in mind, we have performed preliminary experiments combining micro-injection perturbation with the point visiting technology of the DeltaVision system. In this model, a large number of cells were selected for injection. The cells were imaged with DIC and Image Restoration fluorescence microscopy. The micro-injector was loaded and the tip approximated to the center of the microscope field. The previously selected cells were then injected. In our case, we injected a plasmid containing a Yellow Fluorescent Protein (YFP) chimeric protein under constitutive promoter control.

The cells were then visited and re-imaged within ten minutes. By that time, it was evident which cells would survive the injection. In particular, we observed that cells that became detached from the substrate had a high likelihood of mortality. With the remaining cells, a point visiting experiment was initiated as described in the previous section. In early tests, we have observed that less than 10% of the injected cells survived the micro-injection. For example, by combining micro-

injection with point visiting, we could easily inject and follow 20-30 cells. Of these, perhaps 2-3 would tolerate the injection. We could then follow up those cells with 3-4 hours of observations. Rather than require 1015 days of experiments to catch these few cells, we could easily perform this experiment in only a half-day.

As we further develop the methodology, entire experiments with sufficient repeats and controls could be accomplished within a single workday by one individual.

Conclusions

Absolute positioning is critical for the study of any three-dimensional structure, but is critical for live-cell imaging. The use of point-visiting technology with and without micro-injection, can dramatically increase the overall efficiency of cell biology experiments. These increases in efficiency can help bring new methods into every cell biology laboratory and greatly enable novel research into functional genomics, pharmacology, and protein kinetics.

References

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