

Multi-Modal Segmentation for Quantifying Fluorescent Cell Cycle Indicators Throughout Clonal Development

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Abstract—Phase and bright-field microscopy are suitable for long-term, high time resolution *in vitro* imaging of proliferating cells. Such image sequences can extend over a period of days or weeks, and have sufficient spatiotemporal resolution to enable automated segmentation, tracking and lineaging of the cells. Fluorescence microscopy offers a more detailed insight into the cellular state, allowing the presence of specific molecular markers to be interrogated. We have developed a combined segmentation, tracking and lineaging approach that allows the phase imaging channel to be enhanced by information from a periodic fluorescence channel. The system is implemented for a two-channel fluorescence system called FUCCI that is used to indicate the timing of cell cycle progression. In combination with a new denoising algorithm, this approach has been applied to time-lapse image sequences showing clonal development for both human lung cancer cells and mouse T-cells. The method resulted in a significant decrease in the error rate of the automated algorithms, as measured in the amount of effort required by a human observer to correct all segmentation, tracking and lineaging results.

Index Terms—cell segmentation, time-lapse microscopy, T-cell lineaging, FUCCI, lung cancer lineaging, fluorescence denoising

I. INTRODUCTION

The development of microscopes with integrated incubation systems has enabled researchers to generate long-term time-lapse movies capturing cell and clone development through multiple mitotic divisions. The study of these movies has wide application in fields such as immunology, developmental and cancer biology, and regenerative medicine. Phase contrast microscopy allows long-term observation of cells *in vitro* with no modification to the cells and less phototoxicity compared to other approaches. Fluorescent imaging can be combined with phase contrast microscopy to periodically capture additional information on cellular state. In particular, the use of the fluorescent, ubiquitination-based cell cycle indicator (FUCCI) allows researchers to measure cell-cycle dynamics, capturing the transition from the G₁ to S phases in real time: cells fluoresce red during the G₁ phase and green during the S, G₂ and M phases [1].

The analysis of image data showing clones of dividing cells requires the cells to be segmented, tracked and lineaged.

Segmentation delineates the cells in each frame. Tracking establishes temporal correspondences between segmentation results. Lineaging establishes parent-daughter relationships. Previously we have developed a system called LEVER (Lineage Editing and Validation) [2] to analyze phase contrast images of dividing cells. Since errors in tracking and lineaging can quickly corrupt subsequent analysis [3], we have designed LEVER so that a human observer can easily identify and correct errors made in the automated processing.

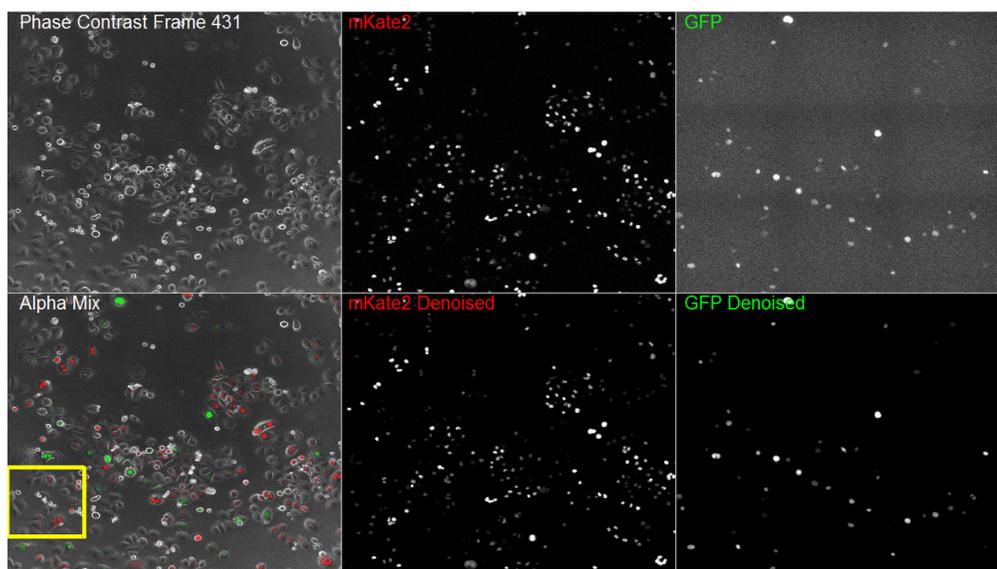
Combining phase and periodic fluorescence information allows us to dramatically improve the accuracy of our segmentation, tracking and lineaging algorithms. For our purposes, a periodic fluorescent signal results either from (1) capturing fluorescence less frequently than phase in order to reduce phototoxicity, or (2) fluorescent signal(s) not being present in a particular cell in a given image frame. When a fluorescent signal is available, it is particularly informative, especially given the difficulty in segmenting complex phase images. Following the approach we developed in [4], we refine the segmentation after tracking and lineaging have been completed. This allows us to incorporate long-term temporal information into the low-level image segmentation task. Our approach here incorporates the FUCCI signals into this segmentation refinement. We also describe a new denoising algorithm that we have found effective for reliably extracting FUCCI signal intensities. We tested our code on two sets of experiments: human lung cancer cells imaged at the German Cancer Research Center, and CD8+ mouse T-cells imaged at the Walter and Eliza Hall Institute. While the results described here are specific to FUCCI signals, the approach is applicable to any combination of phase and periodic fluorescent time-lapse imaging.

Automated segmentation, tracking and lineaging of long-term time-lapse movies of cell and clone development has been the subject of much research in recent years. For example, [5] describes a fully-automated system to segment and track cells. The approach in [6] automatically constructs cell lineages by segmenting and tracking multichannel fluorescent images. A system which incorporates FUCCI is described in [7], which describes an ImageJ plug-in package termed FUCCIJ which displays the FUCCI state on the lineage tree and uses the corresponding cell cycle patterns to predict stem cell fate. LEVER is similar in spirit to these approaches, but differs in several important ways. LEVER is designed such that lineage correction and validation by a

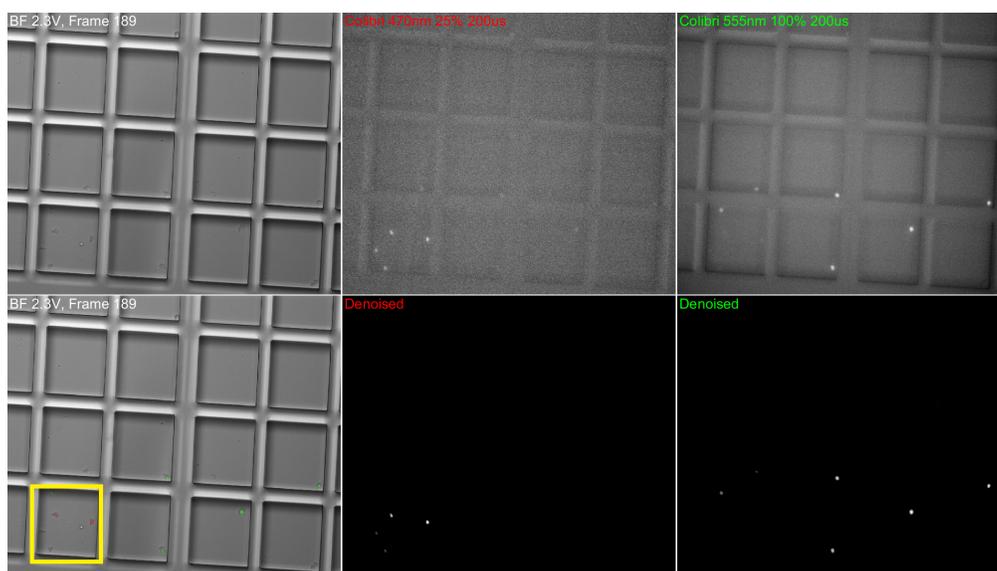
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(a) Human lung cancer cells



(b) Mouse T-cells

Fig. 1: Examples of our denoising algorithm. The top left panels show the phase (a) and bright-field (b) images. Top middle and right show the raw red and green FUCCI channels, and below them are the same images after denoising. The bottom left image shows the FUCCI signals superimposed on the phase/bright-field image. The boxes outline the regions of interest that were cropped and shown in Fig. 2.

human observer is an integral part of the system. Once validated, the corrected lineage is used to refine the segmentation and tracking for subsequent frames, reducing the number of edits required to completely correct and validate a tree. Also, LEVER shows both the phase and intensity of the FUCCI signal directly on the lineage tree.

II. MATERIALS AND METHODS

A. Preparation of Cells

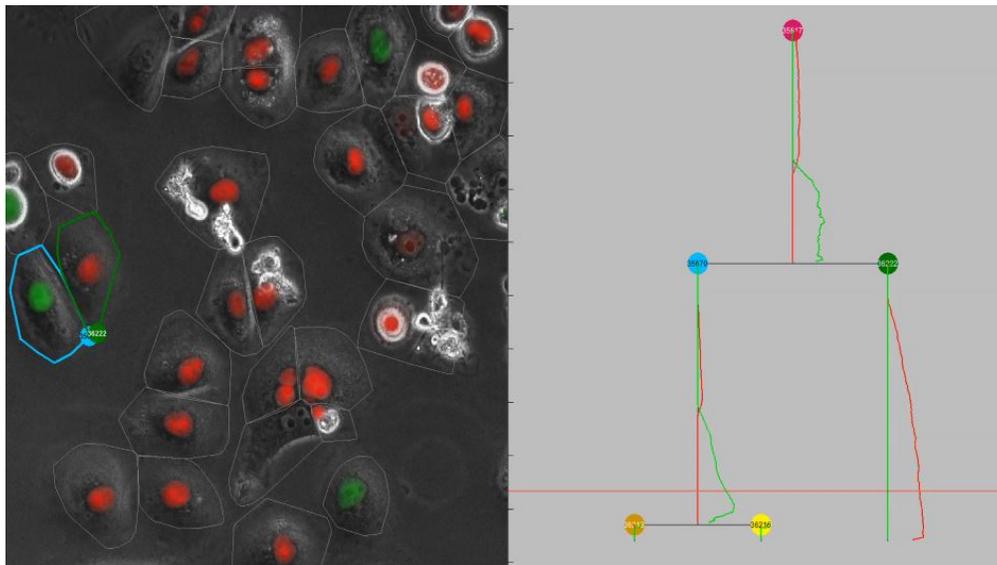
Our first application is with a non-small human lung cancer cell line (H838) transfected with the FUCCI cell cycle sensor. Images were captured every 20 minutes for

the two fluorescent markers (GFP and mKate2) as well as phase contrast. Images were captured as 2711×2705 16-bit TIFF files (Fig. 1a).

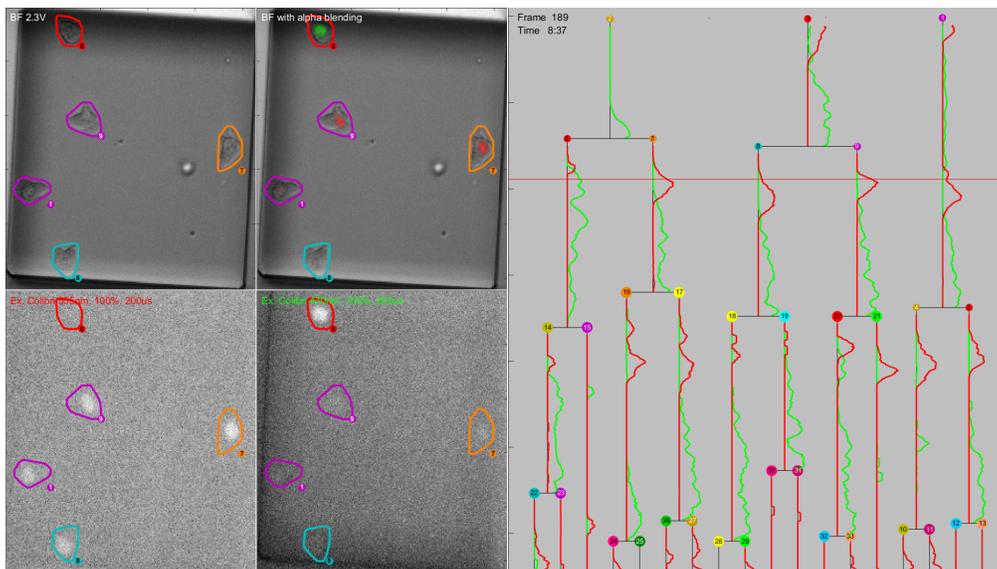
Our second application is with CD8+ T-cells which were purified from lymph nodes of one FUCCI Red/Green female mouse. Four images were captured every 165 seconds for the two fluorescent markers (GFP and DsRed), in transmission, and out-of-focus transmission. Each image was captured as a 1388×1040 TIFF file (Fig. 1b).

B. Image Cropping

Activated lymphocytes are motile, and in order to prevent cell migration outside the field of view, the cells were placed



(a) Human lung cancer cells — 692 frames captured over 10 days



(b) Mouse T-cells — 645 frames captured over 30 hours

Fig. 2: Examples of Fucci signal visualization support in LEVER. For the human lung cancer cells (a) the lineage tree with Fucci signals is shown on the right. On the left the Fucci signal has been alpha-blended with the corresponding phase image. Segmentation and lineage edits update the Fucci signal intensities in real time. As shown with the mouse T-cell example (b), the user can also switch to view the unblended images. The raw bright-field (top left), DsRed (bottom left) and GFP (bottom right) images are shown with colored polygons representing segmentation results. The top right image shows the bright-field blended with the red and green Fucci signals.

in $125 \mu\text{m}$ wells [8]. This gives an additional advantage of preventing cells from forming clusters, but presents a challenge to cell segmentation since the wells are clearly visible in the transmission channel (Fig. 1b). The main challenges of well detection are cells inside the wells, irregular distance between the wells, and an angle between the well edges and the frame boundaries that resulted from the manual placement of a plastic substrate with imprinted wells inside the culturing chamber. The wells were delineated using a combination of edge detection and mathematical morphology [9]. After detection, the image regions internal to each well were extracted and written to separate files for

further processing (Fig. 2b).

C. Denoising, segmentation, tracking, and lineaging

The fluorescent images in the experiments were much noisier than their corresponding phase/bright-field images (Fig. 1) and it was necessary to denoise the images before segmentation. Our denoising algorithm takes as input an image and an initial estimate for foreground and background. The algorithm subtracts the mean and 1–2 standard deviations of the background from the image. A new binary image is created from the pixels that remain positive, and this is fed back into the algorithm iteratively until it no longer changes.

To segment the lung cancer cells, we first applied a standard deviation filter to the phase contrast image. A Gaussian mixture model (GMM) [9] with 3 components was then fitted on the intensities of the filtered images. The pixels were defined as foreground if they were classified into either of the two highest mean clusters. These pixels were merged with the denoised mKate2 and GFP channels and were split into connected components. It was difficult to split these into separate cells based solely on the phase channel, since the cells tended to clump together (Fig. 2a). The FUCCI markers, being nuclear indicators, were smaller than the full cells and so were more easily separated. Therefore, if a single connected component in the combined image overlapped n connected components in the fluorescent images, we assumed that the component represented n cells. We thus used the FUCCI signals, when detected, to split the component into n parts by assigning each pixel to its closest overlapping FUCCI signal. Segmentation of the T-cells was done in a similar manner, but since the FUCCI markers were roughly the same size as the cells (Fig. 2b) they were not used to help split adjacent cells.

Following the initial segmentation, the cells are tracked and lineaged as described in [2] and [10]. Using information from the tracking, lineaging, and fluorescent imaging channels, we identify the number of cells that we are confident should exist in each image frame. A segmentation refinement step [4] is then applied in conjunction with the tracking algorithm to attempt to either split existing segmentations or add new ones in order to obtain segmentations for each cell in every image frame.

III. EXPERIMENTAL RESULTS

To calculate the intensity of the FUCCI signals, we took the denoised red and green channel images and computed the median of all the non-zero points lying within the convex hull of the cell. These were normalized to the range $[0, 1]$ for each track and were displayed graphically on the lineage tree (Fig. 2). The colors were also alpha-blended with the phase images of the cells to make visualization easier. Cell radius and maximum cell velocity parameters were set for the two datasets, and everything else was determined automatically by LEVER.

All of the automated segmentation, tracking and lineaging results were validated by a human observer. Any errors were corrected manually. The LEVER program is designed to make errors easy to identify and correct. For the human lung cancer cells we processed 12 clones containing 68 cells and 5,086 segmentations. The initial segmentation, tracking, and lineaging error rate was 7.3% before we applied the segmentation refinement step that used tracking, lineage, and fluorescence information to automatically improve the segmentation. This is the number of user-provided edits that was required to fully correct any errors. After the application of the segmentation refinement step, the error rate fell to 0.9%.

For the mouse T-cells, we processed 25 clones containing 289 cells and 60,758 segmentations. The initial error rate was

12%. After application of the segmentation refinement step, the error rate fell to 7.5%. Because the time resolution of these images was not sufficient to provide clear discrimination for the tracking algorithm, the segmentation refinement was not as effective as it was for the lung cancer data. Images were captured every 165 seconds, and for future applications involving mouse T-cells we have found that 120 seconds should be the maximum time between images for reliable tracking.

IV. CONCLUSIONS

It is becoming increasingly important to be able to process multi-channel time-lapse image sequences showing the development of clones of dividing cells. Phase and bright-field microscopy allow the cells to be observed long-term with the time resolution sufficient to enable reliable tracking and with minimal phototoxicity and with no modification to the cells. Fluorescence imaging provides a rich source of information on cell state, information that may be available only periodically. The ability to incorporate the fluorescence signal, when available, into the phase and bright-field image processing steps provides an effective means to improve the accuracy of automated algorithms.

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