Electronic supplementary information

Microscopic augmented-reality indicators for long-term live cell time-lapsed imaging

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Materials and methods

Details of the μ-ARI matrix

A single μ-ARI itself only provides the relative position within the scene, not the location within the whole areas of interest. Thus, a matrix of many indicators was used as landmarks. In this study, the indicating markers were arranged in a 10 × 10 matrix form (Fig. 1b). The methodical arrangement of μ-ARIs is for the system to find the desired indicator even if the system were looking another indicator. Because the μ-ARIs should be seen continuously with the microscopic field of view, the size of each indicator should be carefully engineered. Thus, the size of a single μ-ARI was adjusted to guarantee the condition of continuous presence with a field of view of the CCD camera. In this study, the specified width and height of each indicator was 250 μm. There were no margins between each marker, and spaces between the indicators came from its original shape. This matrix of μ-ARI is printed in a film form. The final design of the μ-ARI matrix covers a region of 2500 × 2500 μm^2.

ROI restoration algorithm

The algorithm to restore the ROI was based on the assumption that a user focuses on the layer of the μ-ARIs during the restoring procedure. (Fig. 2) As mentioned in the main article, when the camera captures the images of μ-ARIs, the *reacTIVision* pattern recognition scheme decodes the patters as IDs, positions, and angles of each (Fig. 3a). When a user decides to record a current view as a ROI, a user changes the focus of the objective to the μ-ARI layer, and the software saves one of the recognized indicators' ID, $\mathbf{v}_{now}$ and $\boldsymbol{\theta}_{now}$ as ID of the ROI, $\mathbf{v}_{saved}$ and $\boldsymbol{\theta}_{saved}$. They are used as a reference of the position and rotation of the ROI when the next investigation of the specimen is required.

Because we have the angle of rotation $\boldsymbol{\theta}_{now}$ and $\boldsymbol{\theta}_{saved}$, the angle of rotation of the specimen $\Delta\boldsymbol{\theta}$ is determined directly with respect to the saved ROI position (Fig. 3b).

$$\Delta\boldsymbol{\theta} = \boldsymbol{\theta}_{now} - \boldsymbol{\theta}_{saved} \quad (1)$$

Using the IDs of the indicators and their arrangement within the μ-ARI matrix, the vector $\mathbf{v}_{relative}$ is determined from the starting position of $\mathbf{v}_{now}$ to the starting position of $\mathbf{v}_{saved}$. Finally, the displacement vector $\mathbf{v}$ from the current field of view to the saved ROI (Fig. 3c) becomes

$$\mathbf{v} = -\mathbf{v}_{now} + \mathbf{v}_{relative} + \mathbf{v}_{saved} \quad (2)$$

ROI restoration criteria

In the software instructions, the successful ROI restoration along the x and y direction was defined as 'less than 1 pixel' from the original ROI, for both x and y direction. Once the positions were restored, the field of view of each ROI was captured while focusing on the hemocytometer grids. As a successful restoration range for the x and y position, a successful rotational ROI restoration was also defined. We found that 0.2° was the average range of the computational noise of our system, so the successful rotational criteria was established: When the angle of difference between saved ROI and current field of view became less than 0.2°, the angle of original ROI is successfully restored.

Experimental Setup

Improved Neubauer hemocytometer (DHC-N01 C-Chip, INCYTO, Chungnam-do, Republic of Korea) with a 50 μm grid pattern was used as a virtual 'specimen'. Film-printed μ-ARI matrix was attached underneath the bottom of the grid pattern of this 'specimen'. A single ROI within the 0.0025 mm^2 grid area of the hemocytometer was captured and saved as a reference position for the ROI restoration. Without moving the specimen with respect to the microscopic stage, the saved ROI position was repeatedly restored (n=30) following the software instructions. Comparing the captured grid images with the initial ROI image, the restoration errors of the original center of the ROI were measured. Multiple ROI restoring processes were also simulated with 20 different positions on the hemocytometer grids. By removing the specimen and putting it back on the stage again, not only the location errors, but also the rotational errors could be measured.

Finally, time-lapsed monitoring of the HeLa cell line was conducted. The HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% penicillin streptomycin (PS) and kept in 37°C and 5% CO2 incubator with a 50 ml tissue culture treated polystyrene flask (FALCON, BD Biosciences, California, USA). A film printed μ-ARI matrix was cut with 10 mm margin around the μ-ARI covered area, and the film was adhered beneath of the culture flask of the HeLa cell line with adhesive tape immediately after the initial planting. Using this adhered film of an μ-ARI matrix and our custom software, two separated ROIs of HeLa cell line within the single specimen were captured over 4 hours at one frame per a half hour. and immediately put back into the laboratory incubator after imaging two independent frames of separated ROIs. The incubator was maintained 37°C with a 5% CO_2 concentration during the investigation.

Results and Discussions

Additional Results

To maintain the compactness of the manuscript, the paper excluded some minor experimental results that might not affect the major discussion of the text. In Fig. E1, readers can verify more relocated images for various objective magnifications by μ-ARI matrix.

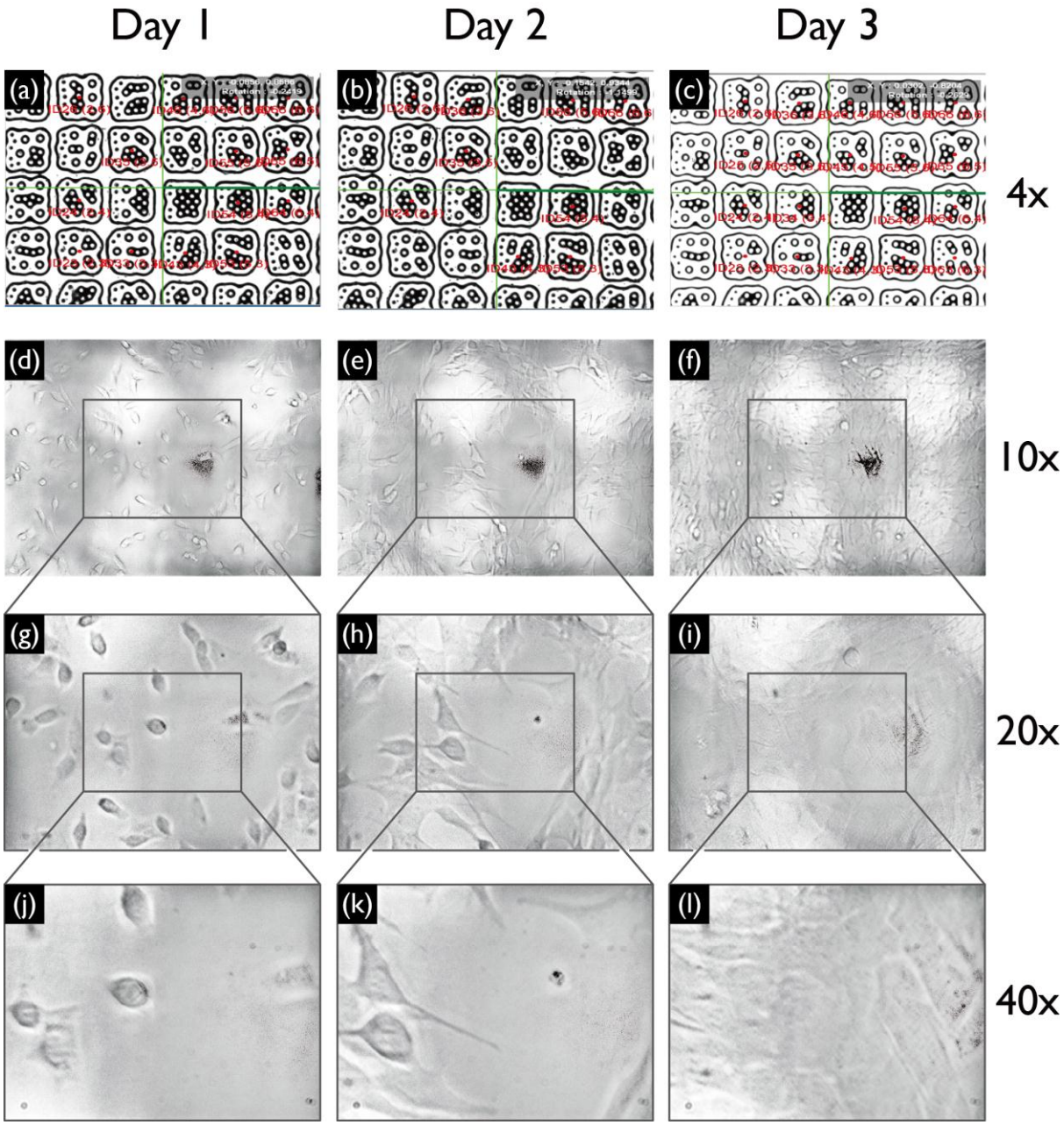

Fig. E1 Time-lapsed images of HeLa cells for 3 days with μ-ARI positioning method. Every 3 day, images were taken with the rate of 1 frame / 24 hour for each objective magnification. Images on the top (a~c) with the 4x magnification are the software screenshot while focused on the μ-ARIs, to recognize the indicators and to find the ROI location. From the day 1 to day 3, the concentration of the HeLa cells are increased, and the locations were successfully restored during the imaging period.

Theoretical resolution limit

Since the software analyzes the pixelized image pattern as a reference map for the ROI, the order of the restoration accuracy was quantized with the pixel resolution of the captured image. In our case, we had a 1600 × 1200 μm^2 imaging area from a 4x objective lens field of view, with a 1024 × 768 imaging resolution. Assuming that the software returns the target position with a single pixel, the theoretical resolution limit of the method becomes

$$\text{Resolution limit} = \frac{\text{Width(Height) of microscopic field of view}}{\text{Width(Height) of captured image}} = \frac{1600\mu m}{1024\ \text{pixel}} = 1.5625\ \mu m/\text{pixel} \qquad (3)$$

Human handling errors

A relatively large standard deviation came from the human handling errors since the specimen flask was rotated by hand. Considering that it is tedious to restore the rotation angle of a specimen without an apparatus to hold the specimen, the μ-ARI matrix can provide a reasonable compass, especially for atypical specimen surfaces such as Petri dishes.

Factors that might affect the result

There are some factors that might affect the result of the method. For example, our method works better with slightly brighter than usual lighting conditions as it whites out unwanted particles on the image that interrupt marker recognition. Also, if the focus is out of range, the recognition algorithm will miss the AR markers. However our software's user interface tells users when to focus on AR markers or the specimen and users who are familiar with microscope operations can easily set up those conditions, following the on-screen instructions.

Ongoing research

There are a few issues we are working through. First, because the μ-ARI patterns generated dappling onto the final images of the specimen, the line thickness of the μ-ARIs could be redesigned so that they are much thinner and they would not affect the quality of the final specimen images. The dappling issues are also being compensated by printing more black dots between the μ-ARIs on the original matrix to reduce the brightness differences on the final image. Evenly distributed black dots were neutralized when they are focused out and blurred on the specimen layer, however, more works are needed on this idea. There were also unwanted scatterings caused by the film on which the μ-ARI matrix was printed. As the film had a different refractive index from the cell culturing ground or the air, the image of the specimen was focused less clearly. Thus, ongoing research is being conducted to resolve this refraction problem. One possible approach is to consider printing the μ-ARI patterns under the thin glass or beneath the culturing area itself.