MULTIPLE TISSUE ANTIGEN ANALYSIS BY SEQUENTIAL IMMUNOFLUORESCENCE STAINING AND MULTI-DIMENSIONAL IMAGE ANALYSIS

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ABSTRACT

A novel method for sequential immunofluorescence staining, which, in combination with 3D image registration and segmentation, can be used to increase the number of antigens that can be observed simultaneously in single cells in tissue sections is presented. Visualization of more than one antigen by multicolor immunostaining is often desirable or even necessary, both for quantitative studies and to explore spatial relationships of functional significance. Sequential staining, meaning repeated application and removal of fluorescence markers, greatly increases the number of different antigens that can be visualized and quantified in single cells using digital imaging fluorescence microscopy. Quantification and efficient objective analysis of the image data requires digital image analysis. A method for 3D image registration combined with 2D and 3D segmentation and 4D extraction of data is described.

1. INTRODUCTION

By combining immunohistochemistry with digital image analysis, properties of specifically stained cells in culture or tissue sections can be quantified. The applications include a wide range of fields such as histology and cytology, where quantification of morphological, biochemical, and molecular changes are of great interest. Biological systems are controlled by a very large number of interacting molecules of different kinds. The number of different antigens that can be observed simultaneously using a fluorescence microscope is limited by the specificity of the immunohistochemical detection system, the spectra of the fluorochromes, and the accuracy of the bandpass filters separating the excitation and emission wavelengths.

The basic requirement for visualization of a given antigen, i.e. a protein, a protein complex, or other macromolecules, is the availability of a relevant antibody reagent that, when used as a specific probe on the test preparation, can be identified by direct or indirect labeling with a discernible signal. Many different staining systems producing detectable signals at the location of a specific antibody have been developed since the early days of immunohistochemistry [3]. Systems based on fluorescence are most commonly used for quantitative antigen analysis [4].

Fluorescent labels (introduced directly onto a primary antibody, or on a secondary antibody) in combination with narrow band pass filters for limitation of fluorochrome excitation and emission opens the possibility to quantitatively image several antigens in parallel in a single multiply stained tissue section. Double and triple stains have been used to observe as many as three different antigens simultaneously [5]. Many problems occur when a large number of antigens are stained at the same time. The different primary and secondary antibodies can interact and ‘bleed-through’ of contrasting emission colors as well as autofluorescence of the stained tissue can disturb the obtained images.

We present a novel method where the fluorescence label and primary antibodies are removed after imaging the stained tissue section. This is done without affecting the antigens, and a new set of specific stains can be applied and imaged. By staining, imaging, and washing repeatedly and using digital image analysis, the number of different antigens that can be imaged and quantitatively analyzed simultaneously in single cells in tissue sections increases. We have limited the number of immunohistochemical stains in each step to two, reducing many of the problems that occur when large numbers of stains are used simultaneously. A third, non-immunohistochemical stain is used to visualize the cell nuclei (see Fig. 1). The two immunohistochemical stains are varied between each imaging step by varying the primary antibody. The fluorescence present before any stain is applied, and the stain that is left after each washing step is also imaged. All images are 3D (three-dimensional) image volumes.

The slide is removed from the microscope after each imaging step. In order to detect the same cells after each washing or staining step it is necessary to remount the slide in the same position. Despite great care and use of an au-
tomatic repositioning function of the microscope, the slide is often translated in x-, y-, and z-direction. Automatic and objective quantification of the antigen content requires exact repositioning. Repositioning by registration is commonly used for radiological images [6]. We have used a four parameter rigid registration algorithm [1] for repositioning of digital images of tissue sections. Each staining or washing step results in an image set consisting of three images acquired in parallel without inter image transformation. The image of the cell nuclei in each set is used as a reference image for registration to the first image set automatically. The same translation and rotation parameters can thereafter be applied to the other images within each set.

After registration, a common reference image is created by a 4D to 3D maximum intensity projection (MIP) and the individual cell nuclei are identified by segmentation. Our method is concentrated on analysis of antigen content in the cell nuclei. The main reason for this is our interest in disturbed behavior of the cell cycle in tumors. Many of the proteins involved in the control of transition events in the cell cycle are located and active in the cell nucleus. Other cellular compartments could of course also be analyzed using different segmentation methods.

The segmentation of the individual nuclei is initialized by an algorithm based on the watershed algorithm, [12][9], working on a 2D MIP of the common reference image. Errors in the segmentation process are corrected by the operator, using a set of digital cutting and merging tools. The 2D segmentation is then used directly or extended to an approximate 3D segmentation by logical operations. The relative antigen concentration in each cell nucleus in the tissue sample is evaluated by calculating the mean of the voxel intensities in 3D or pixel intensities in a 2D MIP. The segmentation based on the images of the cell nuclei is used as a template also for analysis of the images of the antigen stains in the fourth, spectral dimension.

2. IMMUNOHISTOCHEMISTRY AND IMAGE ACQUISITION

Thin sections of tumor tissue are adhered to microscope slides, stained, imaged, washed, and restained repeatedly. A more detailed description of the washing and staining procedures than the one below will be published elsewhere.

2.1. Slide preparation and pre-treatment

Routinely fixed and paraffin embedded samples of cervical carcinoma were cut in as thin sections as possible (2–4μm). The sections were adhered to the microscope slides, deparaffinized and cooked in citrate buffer to recover the antigenicity.

To visualize the nuclei, the slides were incubated with 1μM DAPI (4',6-diamidino-2-phenylindole) before mounting of the slides for fluorescence microscopy. Cover slips were applied, but never sealed, since the slides were usually photographed within minutes and then demounted again. The areas of interest were chosen based on DAPI morphology, and images were acquired to document the amount of autofluorescence, i.e., the natural fluorescence often present in tissue samples. The slides were then demounted.

2.2. Immunofluorescent staining

Prior to staining the slides were washed in washing buffer followed by an incubation in blocking buffer to block non-specific binding of the primary antibodies. Thereafter they were incubated with the primary antibodies diluted in blocking buffer for 16 hours. Unbound and non-specifically bound antibodies were removed by extensive washing.

To block non-specific binding of the secondary antibodies the slides were incubated in serum diluted in blocking buffer. The secondary antibodies, labeled with FITC (fluorescein isothiocyanate) or Cy3 (indocarbocyanine), were then added during a 30 minutes long incubation. The slides were then again stained with DAPI and remounted. The same areas that had previously been photographed were located and a new set of images acquired, this time to document the immunofluorescent staining. The slides were then again demounted and washed in washing buffer to remove remaining mounting medium.

2.3. Strip wash procedure

The removal of the previous stain was performed as a two-step process. First, the secondary antibodies and their conjugated fluorophores, as well as some of the primary antibodies were elutriated during a two hours long washing in a strip wash buffer. During the washing the slides were exposed to light in order to photo bleach remaining fluorophores. Secondly, the remaining primary antibodies were denatured through a repeated antigenic recovery.

Fig. 1: Illustration of the different image sets and their relations to one another. Every box represents a volume image of size 1024 x 1024 x 7 voxels.
The next round of staining could then be performed, beginning with a renewed blocking in blocking buffer, and followed by incubation with fresh primary antibodies, as described above.

2.4. Image acquisition

Images of the sections were obtained using a Delta Vision system, produced by Applied Precision Inc. in Issaquah, WA. The system consists of a mercury lamp with a fiber optic illumination system, conventional microscope optics, selective filters for excitation and emission, a selected Zeiss Plan-Apochromatic 63x / NA 1.4 oil immersion lens, and a water-cooled CCD camera.

Fields containing transformed tissue were chosen based on DAPI morphology during the first round of photographing. The built in repositioning system was used to find the approximate same area again after successive rounds of staining. Every selected field was digitally photographed with each of the filter sets for detection of DAPI, FITC, and Cy-3 respectively, resulting in three images. The procedure was repeated seven times with the focal plane moved 0.5μm between each image acquisition. This resulted in a 1024 × 1024 × 7 image volume for each of the three investigated wavelengths. Pixel size in x- and y-direction is 0.11μm. Working in 3D reduces the need of auto focusing procedures which are otherwise essential in automatic quantitative microscopy [2]. The registration algorithm will align the images also in the focal direction (i.e., z-direction). The exposure times were set to take full use of the dynamic range of the camera, while still not oversaturating it. The exposure times were kept the same for all images acquired from any given investigated area, but sometimes varied from area to area. The same sets of images were also acquired before the first staining step as well as after each washing step between subsequent staining steps (see Fig. 1). These images were used for background subtraction and as negative controls of the washing step.

A linear relationship between fluorophore concentration and emitted light can be expected [8], [11]. The response of the camera is also linear [2]. The intensity of the detected signal is therefore expected to be directly proportional to the amount of fluorophore present in the specimen. The result does however depend on many variables that are often interdependent and without a known standard, it is thus only possible to measure relative antigen concentration in each cell of the tissue section.

3. IMAGE ANALYSIS

The image analysis method can be divided into four steps: image registration, 2D and 3D image segmentation, extraction of image data, and data analysis.

3.1. Image registration

The image registration step registers the different image sets to the first image set by matching. Registration is necessary to ensure that the exact same shape and position can be used for objective automatic analysis of each cell in all subsequent image sets. There is no translation or rotation within an image set as the three different image volumes within a set are acquired in parallel, i.e. the first z-slice of each volume is acquired before moving to the next z-slice. There are however translations in x-, y- and z-direction as well as z-rotation between the subsequent image sets (as shown in Fig. 2) since the slide is de-mounted from the microscope for washing or staining between each set. Rotations other than that around the z-axis and non-rigid transformations are considered as small enough to be neglected.

![Fig. 2: Example of images before and after registration. (a) The reference image and (b) the image to be registered. The difference between the two images before (c) and after (d) match. The zero-level is gray. The transformations in this particular example were rotation of -0.2° around the z-axis, translation -13.6 pixels in x-, -60.9 pixels in y-, and 2.1 pixels in z-direction. All images are MIPs of the 3D result and have been contrast enhanced to show the differences.](image-url)

A transformation that maps all voxels in one image to another can be based on image specific landmarks. It is however both difficult and time-consuming to find good landmarks in this type of images. Instead, the matching algorithm used here is based on the intensities of the image voxels. Each image set consists of one DAPI image, one FITC image and one Cy-3 image (see Fig. 1). FITC and Cy-3 may or may not mark different antigens in different image sets. The DAPI stain is however always used to stain the cell nuclei. This means that we have one image in each set that is common to all image sets, and can thus be used for matching based on voxel intensities.
The matching algorithm needs as input the allowed transformations, a measure of the cost for a given transformation (i.e., a measure of the similarity between two images after transformation), and a minimizer that can find the transformation that results in the lowest cost \[1\]. In this application, the matching algorithm allows for rigid translation in x-, y-, and z-direction and rotation around the z-axis. The cost function is based on the image correlation. The correlation between two images \( f \) and \( g \) is described as

\[
\gamma(T) = \sum_{x \in \Omega} f(x) \cdot g(T(x)) \tag{1}
\]

where \( f \) contains the voxel intensities of the reference image and \( g \) the intensities of the image that we want to transform. \( \Omega \) contains all overlapping voxels in the two images and \( T \) is the transformation matrix.

The correlation is sensitive to variations in amplitude of \( f \) and \( g \). Therefore, the correlation coefficient is used instead. The correlation coefficient is defined as

\[
\gamma(T) = \frac{\sum_{x \in \Omega} (f(x) - \bar{f}(x))(g(T(x)) - \bar{g}(T(x)))}{\sqrt{\sum_{x \in \Omega} (f(x) - \bar{f}(x))^2 \sum_{x \in \Omega} (g(T(x)) - \bar{g}(T(x)))^2}} \tag{2}
\]

where \( \bar{f}(x) \) is the average of the intensity of the reference image voxels contained in \( \Omega \), and \( \bar{g}(T(x)) \) is the average intensity of the voxels contained in \( \Omega \) of the image that we want to transform. The correlation coefficient will vary in the interval \([-1, 1]\). Sub-sampling, by using every 128th, 64th or 16th pixel, has been used to speed up the calculations of the cost measure.

The cost function that should be minimized depends on the transformation of one image in relation to another. We have used Powells method \[10\] for minimization. Powells method starts by searching for a direction where it is possible to decrease the cost function. A one-dimensional minimization method will then search for the minimum along a line in that direction. The procedure is repeated until a minimum in all directions is found. Since the image volumes are very 'flat', i.e., the dimensions are \(1024 \times 1024 \times 7\) voxels, the step size for transformation in z-direction is smaller than in x- and y-direction. This is to prevent the volumes from slipping too far away from each other in the z-direction before a good match in x- and y-direction is found. The registered image is resampled using trilinear interpolation. Using this registration method, the DAPI image of each image set is registered to the DAPI image of the first image set. All image parts that are not overlapping, i.e., not common to all volumes, are excluded from further analysis.

### 2.2 2D and 3D Image segmentation

Before segmentation, a common 3D image is created from all the registered images of the cell nuclei. This common reference image is created by a 4D to 3D MIP. For every voxel, the maximum intensity of all the registered images with respect to a global coordinate system is selected. In this way, only one image has to be segmented for every experiment. Once the common volume and its DAPI intensities are found, the individual cell nuclei have to be found and defined by segmentation.

The initial segmentation is always done on a 2D maximum intensity projection along the z-axis of the common DAPI volume. The main reason for this is the need of operator interaction for correction of errors made by the segmentation algorithm. The staining, imaging, and washing process is tedious and rather complicated. A 100% correct segmentation is therefore desirable and a comparably fast step in the full process. Tools for cutting under-segmented and merging over-segmented objects are far more complicated in 3D than in 2D, making user interaction in 3D difficult. The 2D segmentation can thereafter either be used directly or extended to an approximate 3D segmentation as described below.

### 2D segmentation

A method inspired by the watershed algorithm, \[12][9\], was used for separation of clusters of cells and segmentation of the image into cells and background in one step. A 2D grey-level image can be thought of as a topographic relief where the cells with high grey-levels are peaks separated by lower grey-level valleys. The cells can easily be separated from the background by a single grey-level threshold, see Fig. 3(b). It is however not possible to separate clusters of cells from each other using only one threshold since the valleys between the peaks vary in depth. There are also 'false' valleys due to noise and structures within the cells. These valleys are usually small and do not mark the border between two adjacent cells.

The segmentation algorithm starts from the highest grey-levels, or peaks, in the image and places an upper threshold here. Labels are then assigned to all pixels that are 8-connected to a peak and have a grey-level above a lower threshold. The upper and the lower threshold are then decreased by one, and if an unlabeled pixel with a grey-level equal to the upper threshold is found, it is given a new label. The same label is thereafter given to all 8-connected pixels that are unlabeled and have a grey-value greater than the lower threshold. The upper and the lower threshold are decreased by one and the process is repeated.

The upper and the lower thresholds are separated by a constant distance at all times except when the lower threshold reaches a pre-defined threshold for the background. The distance will then decrease for each iteration until also the upper threshold reaches the threshold for the background. The distance decides the minimum grey-level valley that can separate two adjacent cells and reduces over-segmentation due to false valleys that would appear with the standard watershed algorithm. The result of the initial segmentation
Fig. 3: Segmentation. (a) Grey-level image of cell nuclei. (b) Segmentation result if simple grey-level thresholding is used. (c) Result after watershed based segmentation. (d) Result after automatic removal of small objects and user-interactive correction of errors from (c). Each number represents a label.

step when applied to Fig. 3(a) can be seen in Fig. 3(c). This initial segmentation algorithm usually results in both over- and under-segmentation as well as a lot of small noise objects. Small noise objects are automatically removed based on a size threshold. Over-segmented objects are merged by user interaction simply clicking with the mouse on one part of the object and dragging it to its other part. Splitting is done in a similar fashion by introducing a split line and a new label. The final result is shown in Fig. 3(d).

3D segmentation

The 2D segmentation result can either be used as a template for all further analysis on 2D projections on the registered image sets, or it can be extended to 3D. The extension to 3D is created by simple grey-level thresholding of the common 3D volume at the same level as was used for the threshold for the background in the segmentation of the MIP image described above. The 2D labels are then transferred to the 3D volume by extending the 2D segmentation into ‘3D cylinders’ in the z-direction and making a logical AND between the labeled ‘3D cylinders’ and the 3D thresholded image, keeping the labels of the 2D segmentation. This is a fast and simple method, but it is not completely correct as is illustrated Fig. 4. If the cells are overlapping in the z-direction, the labels will be shared by more than one object. In this particular application, the tissue slices are very thin, so this is usually not a problem. Comparisons of different segmentation strategies are presented in Section 4.

3.3. Extraction of image data

The registration step results in a transformation matrix $T$ for every image set and the segmentation step results in a common segmentation template that can be used on all images after transformation according to the transformation matrix $T$. For extraction of image data, the transformation matrix $T$ of each image set is applied to all the images within the set. If 2D segmentation is used, a MIP is created and the pixel intensities are integrated over each of the cell nuclei defined by the 2D segmentation template. If 3D segmentation is used, no MIP is needed, and the voxel intensities are integrated over the cell nuclei defined by the 3D segmentation template. Since the segmentation templates are created from the DAPI images of the cell nuclei, they will find the correct position of the cell nuclei in the tissue sections independent of the fluorescence signal from the other stains. The 3D segmentation template is thus used for extraction of data in 4D when the different stains are analyzed.

3.4. Data analysis

Many different variables affect the staining intensity of a tissue section. Small variations in tissue fixation and thickness, deparaffinization, antigenic recovery, temperature and time at the staining and washing steps, exposure time, etc. all add up and affect the final detected signal. The variability between different tissue sections makes it difficult to compare cells from different tissue sections in a quantitative way. The staining intensities of cells within the same tissue section can however be compared. In order to make comparisons between cells on different slides it is necessary to classify them according to their intensity values relative the other cells on the same slide. We have previously developed an algorithm for calculation of thresholds for objective classification of a cell nucleus as positive or negative for a particular antigen [5]. The choice of threshold is based on the shape of a histogram of the staining intensities. The method has been further developed by Lindblad [7], and a kernel density estimate is used for approximation of the distributions. In cases where the intensity levels are not negative or positive, but rather continuous, a different method for classification is needed.
4. RESULTS

The described image analysis method consists of two fully automatic steps: image registration and extraction of data, with one semi-automatic step in between: image segmentation. We have tested the method on a number of different images of tissue sections stained according to the staining protocol. Comparisons of the different segmentation methods as well as an experiment that was designed to confirm the sequential staining by repeated staining for the same antigens is presented below. Results from experiments where more than two different antigens are analyzed will be published elsewhere.

4.1. Comparison of segmentation methods

Extraction of image data from maximum intensity projections (MIP) of 3D images may seem like a waste of valuable information. The 3D data is needed for repositioning after shifts in z-direction, and contains more information than a MIP. We have used very thin tissue sections and will therefore have very few overlapping cells in the z-direction, as can be seen in Fig. 5.

Segmentation and extraction of data from a MIP is faster than working in 3D, and manual correction of segmentation errors is simplified if 2D is used. A comparison between the mean of the pixel (or voxel in 3D) intensity per cell nucleus after MIP, in approximate 3D (by extension of MIP segmentation as described in Section 3.2) and true 3D segmentation (done by hand) was compared (see Fig. 6). The errors (i.e. deviations from a straight line) are very small when comparing approximate 3D and true 3D segmentation. Greater errors occur when using a MIP. Extending the MIP segmentation to approximate 3D appears to be a good compromise between the speed of MIP and the accuracy of 3D segmentation.

4.2. Application on image data

To test the reliability of the method and evaluate the washing and staining procedures, a test experiment was set up. Only two antigens (called A and B) were stained, but they were stained and washed away three times switching the type of primary antibody between the two stains after each washing step. Thus, the secondary antibody carrying the label (FITC or Cy-3) shifts between the antigens after each washing and staining step. See Fig. 7. This gave us a priori knowledge of the expected result after staining step 2 and 3, since it should be the same as after staining step 1, only switched between stain 1 and 2.

The variability in staining intensity, as was mentioned in Section 3.4, as well as stain remaining after the washing steps resulted in a need for normalization of the intensity data. A simple method for calculation of the normalized mean pixel intensity per cell is

\[
c_{n,i}(s_{2k}) = \frac{c_i(s_{2k}) - \frac{1}{n} \sum_{i=1}^{n} c_i(s_{2k})}{\left(\frac{1}{n^2} \sum_{i=1}^{n} c_i(s_{2k}) - \frac{1}{n} \sum_{i=1}^{n} c_i(s_{2k})^2\right)^{1/2}}
\]

where \(c_{n,i}(s_{2k})\) is the normalized mean value for cell \(i\) from staining set \(s_{2k}\), \(c_i(s_{2k})\) is the mean pixel intensity of cell \(i\) in set \(s_{2k}\) minus the mean pixel intensity of cell \(i\) before staining (i.e. in set \(s_{2k-1}\)). \(n\) is the number of cells on the tissue slide and \(k = 1, 2, 3\). The washed or unstained set \(s_{2k-1}\) provides a measure of the stain that is present before applying a new stain. The remaining stain is a combination of cellular autofluorescence and stain residuals from previous staining steps. The labeled secondary antibody is always included to visualize remaining primary antibody.

Plots of the normalized staining intensities are shown in Fig. 8. To compare the results, cells that were clearly positive for stain A and B were chosen by visual inspection of stain 1 and 2 in set 1. Cells 2 and 13 in Fig. 7 were selected as positive for stain A and cells 10 and 16 were chosen as representative cells positive for stain B. The normalized mean intensity of the cells are plotted in Fig. 8.

The A-positive cell can easily be separated from the A-negative population (set 2, stain 1), (set 4, stain 2) and (set 6, stain 1) as would be expected. Cells positive for B can be
Fig. 7: Experimental setup for test of our methodology. The secondary labeled antibody is included also after the washing steps to show any primary antibody (anti A and anti B) that has not been completely removed. Cells 2 and 13 were selected as positive for stain A and cells 10 and 16 were chosen as representative cells positive for stain B. All images are MIPs of 3D images that have been scaled the same. Only a small part of the full image is shown.

5. DISCUSSION AND FURTHER DEVELOPMENTS

Sequential staining combined with image analysis opens the possibilities for studies of many proteins simultaneously in single cells. Each successful sequential staining will increase the number of examined antigens per cell by two. Our method for quantification of antigen concentration begins with a fully automatic image registration and creation of a reference image followed by semi automatic 2D segmentation. The 2D segmentation is extended to 3D and, finally, 4D image data is extracted and analyzed.

We show in an application on image data that it is possible to stain and wash three times switching primary antibodies and still identify the same cells as positive or negative for a particular antigen.

The method, including matching, segmentation, and extraction of data, works satisfactory, but the quantification methods still need to be refined. The normalization method described above is unstable due to dependence on the number of positive cells in the tissue. In future work, a normalization method based on the image data will be developed. Further developments include testing of the use of mutual information for classification of antigen content. A segmentation algorithm for 3D, and correction tools for 3D segmentation are also under consideration.

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6. REFERENCES


**Fig. 8**: Plots of the normalized mean pixel intensities per cell in the studied tissue section. The solid lines represents the A-positive cells, dashed lines represent the B-positive cells, and thin dotted lines represent the remaining part of the cell population.


