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Sequential Immunofluorescence Staining and Image Analysis for Detection of Large Numbers of Antigens in Individual Cell Nuclei

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Background: Visualization of more than one antigen by multicolor immunostaining is often desirable or even necessary to explore spatial and temporal relationships of functional significance. Previously presented staining protocols have been limited to the visualization of three or four antigens.

Methods: Immunofluorescence staining was performed both on slices of formalin-fixed tissue and on cells in culture. Images of the stained material were recorded using digital imaging fluorescence microscopy. The primary and secondary antibodies, as well as the fluorophores, were thereafter removed using a combination of denaturation and elution techniques. After removal of the fluorescence stain, a new immunofluorescence staining was performed, visualizing a new set of antigens. The procedure was repeated up to three times. A method for image registration combined with segmentation, extraction of data, and cell classification was developed for efficient and objective analysis of the image data.

Results: The results show that immunofluorescence stains in many cases can be repeatedly removed without major effects on the antigenicity of the sample.

Conclusions: The concentration of at least six different antigens in each cell can thus be measured semiquantitatively using sequential immunofluorescence staining and the described image analysis techniques. The number of antigens that can be visualized in a single sample is considerably increased by the presented protocol. Cytometry 47:32–41, 2002. © 2001 Wiley-Liss, Inc.

Key terms: sequential immunofluorescence staining; 3D image analysis; antibody denaturation; antibody elution

In 1941, Albert Coons performed the first successful visualization of an antigen using immunofluorescence (1,2). For many years, fluorescein isothiocyanate (FITC) was the only fluorophore available. In 1957, rhodamine was introduced as an alternative fluorescent label (3). The introduction of narrow-band excitation and emission filters meant that FITC and rhodamine could be studied independently in the same specimen, allowing the exploration of the exact spatial relationship between two antigens (4).

Simultaneous visualization of three or more antigens by immunofluorescence is often desirable. Multicolor immunofluorescence staining is, however, limited by two factors. The first is the spectra of the fluorophores, which have to be separated by band pass filters for the excitation and emission wavelengths. If the spectra of the fluorophores overlap, which is often the case when many fluorophores are used together, it is very difficult to discriminate between signals originating from different fluorophores. The second factor limiting multicolor immunofluorescence staining is the availability of primary antibodies of different origins. The majority of commercially available antibodies are either of rabbit or mouse origin, limiting the number of possible combinations of primary antibodies.

Several techniques have been developed to overcome the lack of primary antibodies from different species. The primary antibodies can be conjugated directly with the fluorophores, so that secondary antibodies directed against primary antibodies of a specific species origin are no longer needed. Unfortunately, the successful detection of most antigens requires the amplification of the signal that is achieved when a secondary fluorophore-conjugated antibody is used, and fluorophore conjugation can change the affinity of the primary antibody. Two primary antibodies from the same species can be used if one of the various blocking techniques using Fab fragments is employed (5) or if the first secondary antibody is replaced by fluorophore-conjugated Fab fragments (6).

The two first authors contributed equally to the presented work. FE developed the staining protocol and CW developed the image analysis procedures.

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An entirely different approach used occasionally in immunostaining is elution of the first applied primary and secondary antibodies using a low pH buffer leaving only the colored end product (7). After elution, another round of staining can be performed, using a second primary antibody from the same species as the first together with a differently colored end product. However, fluorophores cannot withstand elution. More recently, denaturation of the primary antibody by heating of the sample has been used successfully to destroy the first applied primary antibody (8). Again, the method used can only be applied to traditional immunostaining, not immunofluorescence.

We have developed a novel protocol for sequential immunofluorescence staining (SIFS) based on a combination of elution and denaturation of both primary and secondary antibodies, as well as of the fluorophores. The procedure allows us to stain the same tissue slice at least three times, allowing the study of at least six antigens in one slice of tissue. Different primary antibodies from the same species could be applied at different staining rounds, overcoming the limited availability of primary antibodies from different species without the need for blocking the first applied primary antibody. Interesting parts of the tissue were photographed between each round of staining. Through digital registration of the images from each round of staining, an image of the studied area that contained all the information on how each antigen was distributed could be created. After semiautomatic identification of individual cell nuclei in the acquired images, the staining intensity of each stain in each cell nucleus in the tissue slice could be extracted. Based on the relative staining intensities, each cell was classified automatically as positive or negative for each of the investigated antigens. The morphology of the tissue could be visualized using standard HTX-eosin staining after the completion of the SIFS procedure.

MATERIALS AND METHODS

Preparation of Tissue

Tissue sections from routinely fixed and paraffin-embedded samples of cervical carcinoma or carcinoma of the prostate were used to test and optimize the proposed protocol. The tissue sections were cut at a thickness of 2–4 μm. Thin sections are necessary in order to minimize image blurring from multiple layers of cells. The sections were incubated overnight at 47°C to adhere to SuperFrost Plus microscope slides (Menzler Gläser, Braunschweig, Germany). The sections were stored at -20°C and stepwise deparaffinized in graded alcohols prior to staining. Antigenic recovery was performed through microwave cooking for 2 × 5 min in a 0.1 M citrate buffer, pH 6.0.

To visualize the cell nuclei, the tissue sections were incubated in a 10-μM 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI) solution for 5 min. The sections were washed briefly in washing buffer (0.05 mM TRIS-HCl, pH 7.6, 0.3 mM NaCl, and 0.02% Tween 20) and mounted in DABCO mounting medium (25 mg/ml 1,4-diazabicyclo[2,2,2]octane and 0.1 × phosphate-buffered saline [PBS] in spectroglycerol, pH set to 8.6 using HCl). An approximately 170 μm thick (no. 1.5) coverglass was used to optimize the optical conditions for 3D microscopy.

The areas to be studied were chosen based on DAPI morphology. They were photographed digitally before the first round of staining, making it possible to measure separately the amount of autofluorescence emitted from the sample for all filter sets. Filter sets optimized for detection of DAPI, FITC, and Cy3 from Chroma Technology (Brattleboro, VT) were used. The images were acquired using a Delta Vision system (Applied Precision, Seattle, WA) and equipped with a cooled monochrome CCD camera (Photometrics, Tucson, AZ). A Zeiss Plan-Neofluar 63×/NA1.30 lens was used (Carl Zeiss GmbH, Oberkochen, Germany), resulting in a pixel size of 0.1058 μm and an optical resolution of approximately 0.2 μm in the x and y directions. In order to get a 3D image of the studied area, seven z sections 0.5 μm apart were imaged, making the final resolution of the 3D volume in the z direction 1.0 μm. The initial focus was found by visual inspection.

After the background had been documented, the slides were demounted carefully. Each time the slides were demounted, they were washed for at least 15 min in washing buffer before being further processed.

SIFS

Sequential staining on tissue sections has been performed using a variety of primary antibodies. For optimization of the protocol, the mouse monoclonal antibody (mAb) 6E6 against cyclin A (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) and the rabbit polyclonal antibody sc-528 directed against p27 (Santa Cruz Biotechnology, Santa Cruz, CA) were used. In addition, the rabbit polyclonal antibody H-432 against cyclin A (Santa Cruz Biotechnology) and the mouse mAb K25020 against p27 (Transduction Laboratories, Lexington, KY) were used for the crossover experiments. All secondary antibodies were of donkey origin (Jackson ImmunoResearch Labs, West Grove, PA). The used secondary antibodies included an FITC-conjugated anti-rabbit antibody, a Cy3-conjugated anti-mouse antibody, and a biotin-conjugated anti-mouse antibody. The secondary antibodies were dissolved in blocking buffer with 4% donkey serum. Detection of the biotin was performed using Cy3-conjugated streptavidin (Amersham Life Sciences, Little Chalfont, United Kingdom). The streptavidin was diluted in PBS.

To block nonspecific binding, the tissue sections were immersed in blocking buffer (1% bovine serum albumin [BSA] and 0.5% Tween 20 in PBS) for 30 min. The primary antibodies were then applied. The slides were covered by coverglasses and left overnight at room temperature in a moisture chamber. The tissue sections were washed in washing buffer 3 × 15 min. The tissue sections were immersed in blocking buffer a second time for 30 min, this time using blocking buffer with an addition of 4% donkey serum. The tissue sections were incubated with the secondary antibodies for 30 min, followed by another round

of washing in washing buffer 3 × 15 min. Whenever a biotin-conjugated secondary antibody had been used, the slides were incubated with biotin-Cy3 for 30 min and washed again as described above. Finally, the slides were incubated with DAPI and mounted as described.

After photographing the result of the first round of staining, the antibodies were disposed of using elution, denaturation, or any combination of these (see below). Elution of previously applied secondary and primary antibodies was performed using primarily a 0.1-M lysine buffer, pH 2.0 (7). Denaturation of the applied antibodies was performed in exactly the same way as the antigenic recovery; the slides were cooked in a citrate buffer in a microwave oven (8).

After the denaturation and/or elution of the stain, the areas of interest were again photographed to check the remaining levels of the previously applied antibodies and fluorophores. To measure the amount of remaining fluorophores, the imaging was performed simply without further treatment. To measure both the remaining primary antibodies and the remaining fluorophores, the tissue section was immersed again in blocking buffer with 4% donkey serum, incubated with the same secondary antibody as used previously, and washed again as described above. Any remaining primary antibodies would then cause fluorescence by attracting the newly applied secondary antibodies, and thus be detected.

The subsequent rounds of staining or stain removal were performed in the same manner as described above for the first staining round. Each round of staining or stain removal resulted in one image volume for each of the used (or recently removed) fluorophores and one for the DAPI stain. DAPI was used as a reference stain in the image analysis and therefore had to be reapplied in each round of staining because the stain removal procedure also removed the DAPI stain. Thus, the background fluorescence before the first staining as well as any remaining fluorophores or primary antibodies after each stain removal were documented together with the staining results. By staining the slides for hematoxylin-eosin after the sequential immunostaining procedure, the morphology of the investigated tissue could be investigated.

Image Analysis

In order to quantify the fluorescence emitted by each investigated nucleus at each step of the staining process, the acquired volumes were analyzed using the image analysis methods described previously (9). Expression that extends beyond the nucleus could be analyzed by segmentation of the cytoplasms. This was, however, not investigated in this study. In summary, the image analysis methods can be divided into four steps: image registration, 2D and 3D image segmentation, extraction of image data, and data analysis.

Image Registration

After each round of staining or stain removal, the slides were placed in the microscope and photographed. One monochrome image with each of the filter sets for detection of DAPI, FITC, and Cy3 was acquired before moving the focal plane to the next z-position. This was repeated seven times until one 1024 × 1024 × 7 voxel image volume for each of the investigated wavelengths was created. Each time the slides were reinserted in the microscope, they were repositioned using the automatic repositioning system of the DeltaVision system. The repositioning was not perfect and translation in the x-, y-, and z-direction, as well as z-rotation between the subsequent image sets, remained. Typically, a 5–10-pixel translation in the x- and y-direction, a 1-pixel translation in the z-direction, and a 1–2° z-rotation appeared between subsequent image sets. Exact repositioning, or registration, of the images was necessary for the automatic steps of further analysis based on individual cells.

Because each image set contained a DAPI image and the DAPI morphology did not change between subsequent rounds of staining or stain removal, the DAPI images were used for registration of the whole image set. The DAPI image of each image set was registered automatically to the DAPI image of the first image set using gray-level matching in 3D (9). A smaller iteration step size was used in the z-direction than in the x- and y-direction because of the flat shape of the image volumes (1024 × 1024 × 7 pixels) and the smaller translation error in the z-direction. The transformation matrix of the best registration was then applied to the FITC and Cy3 images within the same set.

2D and 3D Image Segmentation

Once all image volumes were registered, the cell nuclei had to be identified by segmentation so that the fluorescence signal could be extracted. Before segmentation, a common 3D reference image was created from all the registered DAPI images of the cell nuclei. For every voxel, the maximum intensity of all the registered images with respect to a global coordinate system was selected. In this way, only the reference image had to be segmented for every experiment. In other words, for an experiment with three sequential triple stainings, resulting in 18 image volumes, only one image had to be segmented.

A method inspired by the watershed algorithm (10,11) was used for separation of clusters of cells and segmentation of the image into cells and background in one step as described in detail (9). The initial segmentation using this method was always done on a 2D maximum intensity projection along the z-axis of the reference image. This fully automatic initial segmentation results in both oversegmentation and undersegmentation in areas of closely clustered nuclei. Errors were corrected manually using a digital editing tool, making the segmentation step semiautomatic. The manual correction is a minor part of the otherwise fully automatic process because only a single 2D segmentation is needed for complete analysis of each full sequential staining experiment. The extension to 3D was created by gray-level thresholding of the common 3D volume at the same level as was used for the threshold for the background in the 2D segmentation. The 2D labels were transferred automatically to the 3D volume by ex-

tending 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.

Extraction of Image Data

The registration step resulted in a transformation matrix for every image set and the segmentation step resulted in a common segmentation template that could be used on all images after transformation according to the transformation matrix. For extraction of image data, the transformation matrix of each image set was applied to all the images within the set. The voxel intensities were thereafter integrated over each cell nucleus defined by the 3D segmentation template. Because the segmentation templates were created from the DAPI images of the cell nuclei, they found the correct position of the cell nuclei in the tissue sections independent of the fluorescence signal from the other stains.

Data Analysis

A linear relationship between fluorophore concentration and emitted light can be expected (12,13). The response of the camera is also linear (14). The intensity of the detected signal can therefore be expected to be directly proportional to the amount of fluorophore present in the specimen. However, many variables affect the staining intensity of a tissue section. Variations in tissue fixation and thickness, deparaffinization, antigenic recovery, temperature and time at the staining and washing steps, and exposure time all affect the final detected signal. The variability between different tissue sections makes it difficult to compare quantitatively cells from different tissue sections. The staining intensities of cells within the same tissue section can be compared. In order to make comparisons among cells on different slides, it is necessary to classify them according to their intensity values relative to the other cells on the same slide. We have developed an algorithm for the calculation of thresholds for objective classification of a cell nucleus as staining positive or negative for a particular antigen (15). The choice of threshold is based on the shape of a histogram of the staining intensities. The method was developed further by Lindblad (16) and a kernel density estimate was used for approximation of the distributions. Based on the thresholds given by maxima in the second derivative of the kernel density estimate, a fuzzy class membership value was found for each cell (17). Cells were classified as strongly negative, negative, neutral, positive, or strongly positive.

RESULTS

During the development of the described protocol for SIFS of routinely fixed and paraffin-embedded material, a series of experiments were performed. Parameters for elution and denaturation were adjusted to optimize the removal of previously applied antibodies without destroying antigenicity of the tissue sections. The level of success was evaluated using the described image analysis techniques. All experiments were performed two or more times to ensure reproducibility.

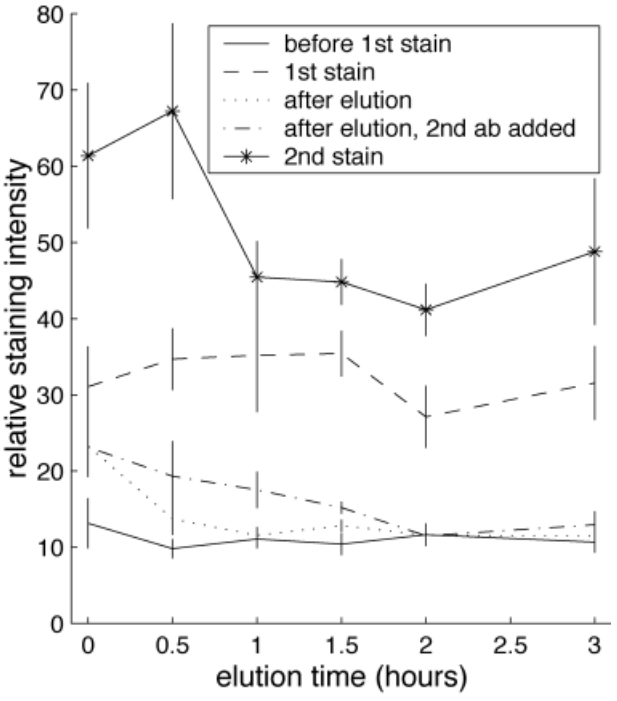


FIG. 1. Stain removal by elution. The staining intensity of a subset of representative positive cells (found by inspection of the slides after the first staining step) from six slides were studied before staining (but with secondary antibodies added), after application of the first stain, after elution for 0, 0.5, 1, 1.5, 2, or 3 h, after the addition of the new FITC-conjugated secondary antibody, and after the application of the new primary and FITC-conjugated secondary antibodies. After 1.5 h, the majority of the previously applied primary and secondary antibodies had been removed. However, when the FITC-conjugated secondary antibody was reapplied, previously strongly staining nuclei still emitted a weak fluorescence. This indicated that a small subset of the primary antibodies remained bound to their antigens, even after 3 h of elution. The antigens were not destroyed by the treatment and the staining intensity increased for the second round of staining, indicating that the antigens are made more available to the antibodies after elution. A very high staining intensity appeared if the elution was shorter than 1 h, probably due to formation of complexes of primary and secondary antibodies.

Elution for Removal of Applied Primary and Secondary Antibodies

In order to study whether elution using a glycine buffer was sufficient to remove all previously applied antibodies, as well as the fluorophores conjugated to the secondary antibodies without disturbing the antigen, slides were washed in glycine buffer, pH 2.0, for 0, 0.5, 1, 1.5, 2, and 3 h. The results are shown in Figure 1. After 1–2 h, the majority of the previously applied fluorophores were removed. However, when the secondary antibody was reapplied, previously strongly staining nuclei still emitted a weak fluorescence, even after 3 h of elution. This indicated that a subset of the primary antibodies remained bound to their antigens.

The primary antigens (cyclin A and p27) were not destroyed by the elution and the staining intensity actually

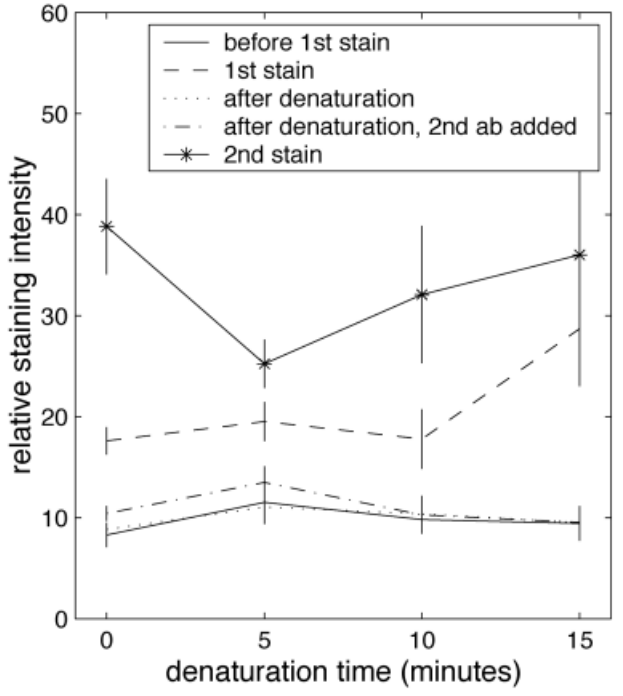


FIG. 2. Stain removal by denaturation. As elution did not remove all the primary antibodies, an attempt was made to denature the remaining antibodies. The staining intensity of a subset of representative positive cells (found by inspection of the slides after the first staining step) from five slides was studied before staining, after application of the first stain, after elution for 2 h followed by denaturation for 0, 5, 2 × 5, or 3 × 5 min, after addition of the new FITC-conjugated secondary antibody, and after application of the new primary and FITC-conjugated secondary antibodies. When the 2 h elution was followed by denaturation for 2 × 5 min, all the primary antibodies were removed or destroyed.

increased in the second round of staining, indicating that the antigens might be more readily available after elution. A very high staining intensity after the second staining round was noted if the elution time was shorter than 1 h, probably due to remaining secondary antibodies binding the subsequently applied primary antibodies. Elution did not fully remove applied biotin-streptavidin-Cy3 complexes. Large aggregates of streptavidin-Cy3 remained even after 6 h of elution (data not shown).

Denaturation Can Destroy Remaining Antibodies

As elution did not remove all the primary antibodies, an attempt was made to destroy the remaining antibodies through heat denaturation. Slides that had been stained and exposed to elution for 2 h were denatured for 0, 5, 2 × 5, or 3 × 5 min. As described previously, 2 × 5 min was sufficient time to denature all the primary antibodies (8; Fig. 2).

Denaturation Alone or Followed by Elution Cannot Remove Fluorophores

The efficiency of denaturation alone, or denaturation followed by a shorter elution, was tested to see whether it would be equally efficient in removing the primary and secondary antibodies as a 2 h elution followed by a 2 × 5 min denaturation. Denaturation alone failed completely in removing the fluorophores (data not shown) and it also seemed to lower considerably the efficiency of a follow-up elution (data not shown).

Crossover Experiments

A crossover experiment was designed to test if the stain is washed away completely without affecting antigenicity, as well as to test whether the same cell will be classified as positive/intermediate/negative if the same stain is applied again. One crossover experiment consisting of three rounds of staining is shown in Figure 3. Only two antigens (cyclin A and p27) were stained throughout the experiment, but two different primary antibodies were used for each antigen. One of the antibodies was of mouse origin and one was of rabbit origin. After each stain removal, the antibody used to detect each of the antigens was switched. Thus, the secondary antibody carrying the fluorophore (FITC or Cy3) shifted between the antigens after each staining step. This setup gives a priori knowledge of the expected result of the second and third rounds of staining, because the result should be the same as after the first round of staining, but in the opposite fluorophore channel. The labeled secondary antibody was reapplied after each stain removal to visualize the remaining primary antibody. Thereby, the removal of the previously applied antibodies could be measured. The experiment provided us with a method to show that the antigenicity of the investigated antigens was not destroyed by elution or denaturation and that the antibodies and fluorophores could be removed satisfactorily after each of the two first rounds of staining.

Any residual fluorescence from previous staining steps was recorded by imaging the specimen after each stain removal. The exposure time was kept constant throughout the experiment for all images acquired with the same filter set. Autofluorescence, as well as residual fluorescence remaining after the stain removal step, could be removed from the subsequent staining step by simple pixel-wise subtraction. The relative antigen concentration per cell was approximated as the mean pixel intensity under the 3D segmentation template as described above. Each cell was then classified as positive (green), weakly positive (cyan), neutral (blue), weakly negative (magenta), or negative (red) using the described classification methods. The classification results are shown in Figure 4 together with the images of the stained cells (after subtraction of the corresponding residual images). The images were scaled for optimal visualization of gray levels. As shown in Figure 4, the cells are classified the same way after each of the three staining rounds for cyclin A (with two minor changes from neutral to weakly positive). This shows that the antigenicity is kept after the subsequent staining steps and that the classification is not affected by previous staining steps. The variations of the classification results are larger when it comes to the p27 stain. The results are similar after the first and third staining steps, when the rabbit polyclonal primary antibody was used, compared with the second staining step, when the mouse

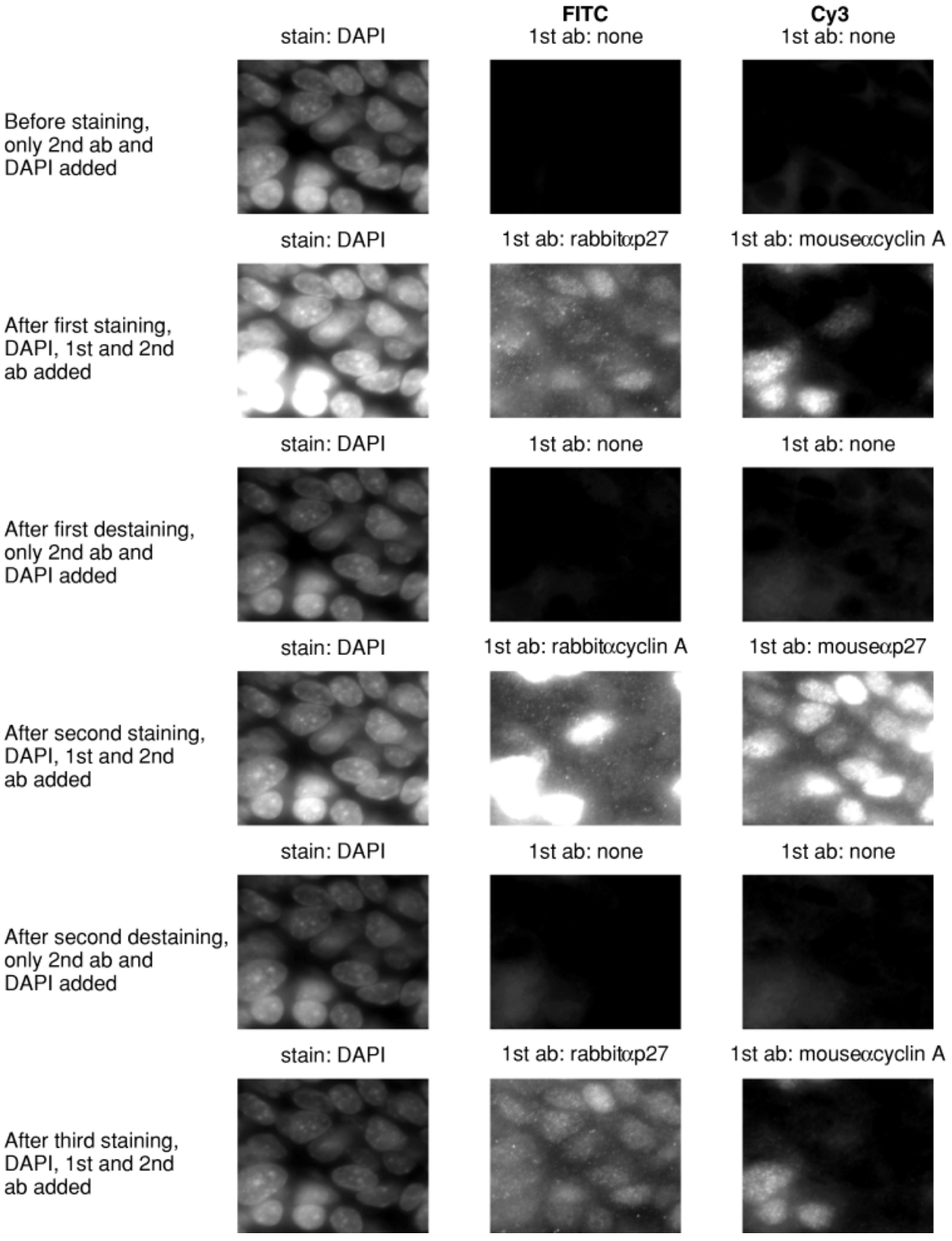
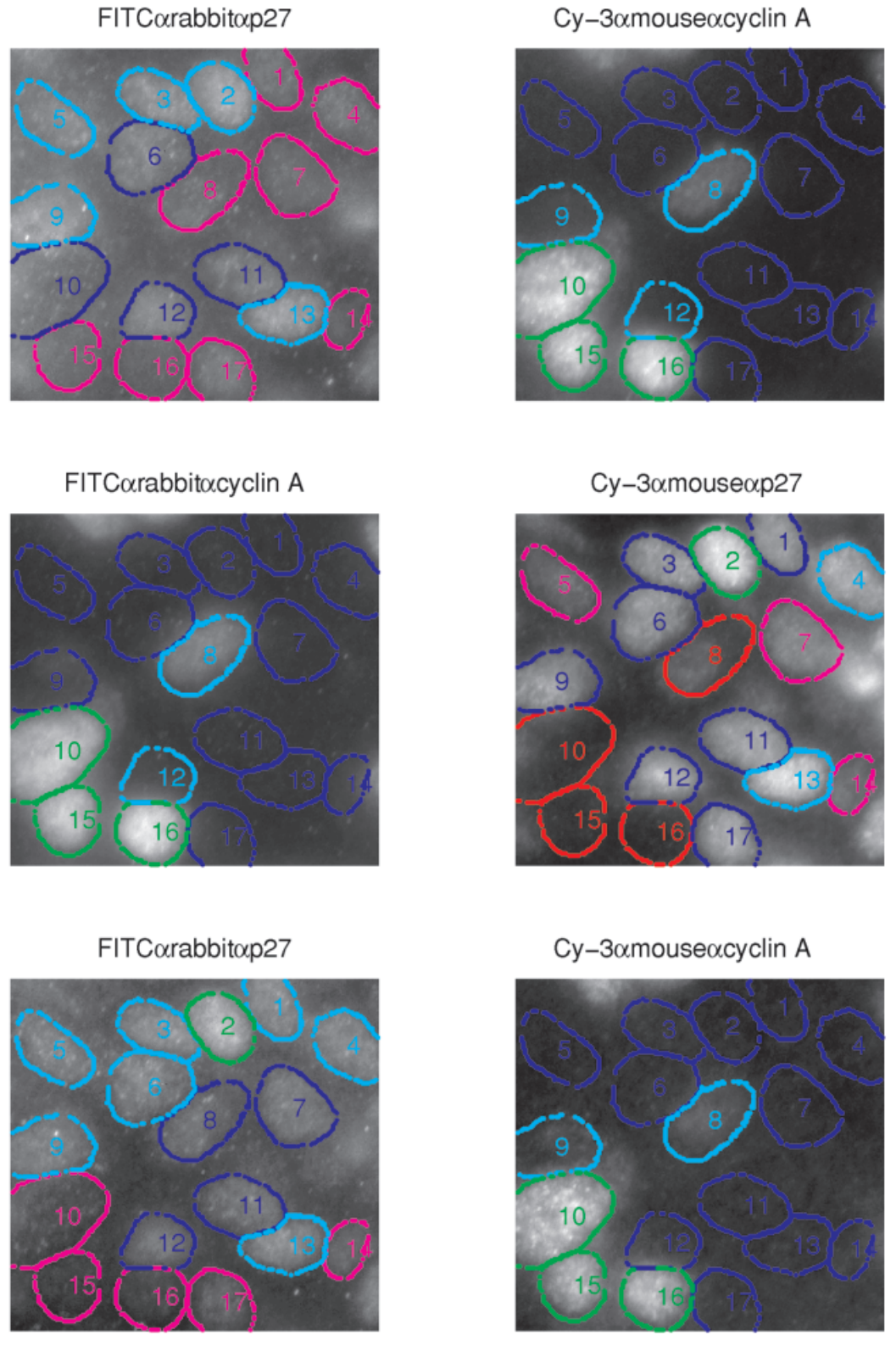


FIG. 3. Set up of the crossover experiment. In each staining or washing step (horizontal rows), the tissue was stained with DAPI and the secondary antibodies FITCαrabbit and Cy3αmouse were applied. The primary antibodies were excluded after each stain removal step and exchanged before the next round of staining. All images are projections of 3D images (explaining the blur). They were acquired using the same exposure time and were subsequently scaled using the same gray-level interval. Histogram equalization or other image enhancement (which would have improved the print quality) was avoided in order to visualize the true variation in fluorescence signal. Only a small part of the full registered image set is shown.


FITCαrabbitαp27
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FITCαrabbitαcyclin A
Cy–3αmouseαp27
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Fig. 4.

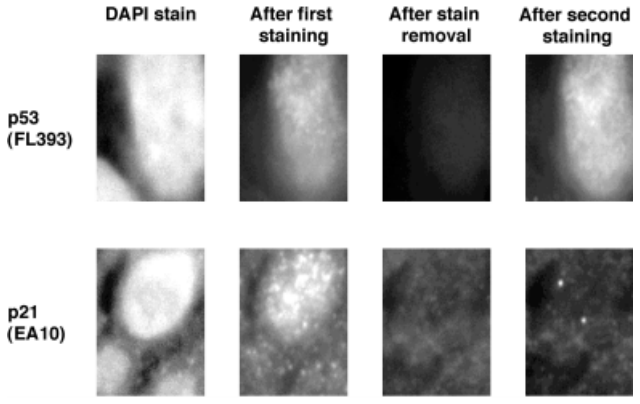

FIG. 5. Two different reactions to elution and denaturation. The antibody FL393 against p53 is well removed by elution for 2 h and a 2 × 5-min denaturation and p53 retains its antigenicity. The majority of the tested antibodies and antigens reacted similarly. p21reacted entirely differently. It lost all of its antigenicity through the elution. It is the only antigen to exhibit this type of behavior. Each set of three images was acquired using the same exposure time. The images were handled exactly the same way with respect to contrast and brightness settings.

monoclonal primary antibody was used. The most likely explanation is the differences in the specificity of the used primary antibodies.

The conclusion of the described crossover experiment is that previously applied stains can be removed at least twice and that at least two rounds of stain removal can be performed without necessarily losing the antigenicity of the investigated antigens. This opens the possibility to stain one slice of tissue for at least six different antigens, using two primary antibodies of different species origin in each staining step.

Some Antigens Lose Their Antigenicity During Elution

The results presented above were acquired using antibodies directed against cyclin A and p27 because these proteins are located primarily in the cell nucleus and because cells generally contain either cyclin A or p27, but not both, which simplified the verification of the method. Other antibodies were also tested in order to see whether a 2 h elution and a 2 × 5 min heat denaturation would affect them and their antigens similarly. Among the tested antibodies, two strikingly different patterns could be distinguished (Fig. 5), which are illustrated by the p53 antibody FL-393 (Santa Cruz Biotechnology) and the p21 antibody EA10 (Oncogene Research Products, Darmstadt, Germany). The antibody detecting p53 was removed or destroyed sufficiently by the treatment and it stained even stronger in the second round of staining than in the first, just like the previously described antibodies against cyclin A and p27. Other antibodies exhibiting the same behavior included another antibody directed against p53 (DO1, Santa Cruz Biotechnology), an antibody against MDM2 (SMP14, Santa Cruz Biotechnology), an antibody against cyclin E (HE12, Santa Cruz Biotechnology), and an antibody directed against p57 (C-20, Santa Cruz Biotechnology). On the other hand, antibody EA10 against p21 did not stain at all in the second round, indicating that the epitope on p21 that it detects was destroyed by elution. Thus, 9 of the 10 tested antibodies (five of the six tested antigens) may be used in a second or later round of staining, whereas the antibody detecting p21 must be used in the first staining round.

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FIG. 4. Results of the image analysis and classification procedures. Each cell was classified as positive (green), weakly positive (cyan), neutral (blue), weakly negative (magenta), or negative (red) using the classification methods described in the Materials and Methods. Notice how the cyclin A stain results in almost the exact same classification of the cells after each of the staining steps. The variation in classification is larger for the p27 stain, but this can be explained by differences in the reactivity of the used primary antibodies, because the first and third rounds of staining were almost identical.

DISCUSSION

The results presented show that primary and secondary antibodies, as well as fluorophores, can be removed from a routinely fixed and paraffin-embedded tissue slice. The procedure is based on the elution of antibodies and fluorophores, followed by the denaturation of any remaining primary antibodies. Thus, a tissue slice can be stained repeatedly, allowing the detection of a large number of antigens. Our method for quantification of antigen concentration begins with a fully automatic image registration and the creation of a reference image followed by semiautomatic 2D segmentation. The 2D segmentation is extended to 3D and, finally, image data are extracted and analyzed.

The presented SIFS technique requires the complete removal of previously applied fluorophores and antibodies without disruption of the antigenicity of the sample. We used elution and denaturation, but other means to achieve the same or better results probably exist. The presented protocol is capable of a complete removal of fluorophores and antibodies when only visual inspection is used to evaluate the results. Occasionally, enough primary antibodies remain to be detected when a highly sensitive CCD camera is used. The weak signal emanating from remaining fluorophores and antibodies could be subtracted from the following staining round using the image analysis techniques and the experimental setup described. Another slight drawback of the proposed protocol is that the elution tends to increase nuclear background fluorescence.

Elution and denaturation increase the antigenicity of the tissue for the majority of the tested antigens. The second round of staining is generally stronger than the first and the presented experiments (Figs. 1, 2) indicate that this is not an artifact due to remaining primary and secondary antibodies after the first staining round. The most probable cause of the phenomena is that elution and denaturation actually improve the antigenicity of the tissue by improving the previously performed antigenic recovery. Antigenic recovery performed for 3 or 4 × 5 min instead of the standard 2 × 5 min sometimes yields stronger staining (data not shown).

During the development of the presented procedure, a high variability of staining intensity in different tissues, as well as in material fixed differently, was noticed. It is possible that the described protocol has to be adapted to work with material from other tissues than cervical carcinoma and with material that is sectioned and fixed differently. However, the presented protocol has been used successfully for at least two rounds of staining even on cells grown in monolayer culture and fixed for 1 h in 10% formalin (Hanna-Stina Martinsson, personal communication).

Before designing a full SIFS experiment, the antibodies and their antigens have to be tested extensively in order to evaluate their reaction to elution and denaturation. We have seen two types of reactions. The majority of antibodies are removed readily and their antigens retain their antigenicity (see p53 in Fig. 5). The second type of reaction is illustrated by p21 (Fig. 5). The primary antibody is removed easily, but the elution destroys the antigenicity of p21. Antigens exhibiting this behavior can only be stained in the first staining round. Primary or secondary antibodies that are not removed by elution represent a possible third type of reaction to elution and denaturation. These antibodies can only be applied in the last round of staining. The amount of remaining primary antibody that can be accepted, i.e., the distinction between the first and the third type of reaction, may vary depending on the fluorescence detection system and the availability of image analysis tools to remove any lingering signal from subsequent images.

Each new secondary antibody and each new fluorophore will also require testing before usage. To ensure optimal results, elution pH and duration, denaturation temperature and duration, and staining order may have to be optimized for each individual SIFS experiment.

Several alternative methods to remove or destroy primary antibodies and/or fluorophore-conjugated secondary antibodies have been presented. Elution has been performed with varying degrees of success using glycine buffer (7), dimethylformamide (18), glycine and dimethylformamide in combination with electrophoresis (19), $KMnO_4$-H_2SO_4 (20), and HCl (21). Shorter denaturation at a higher temperature (130°C) can be highly efficient if the tissue is properly shielded by immersion in glycerin for 5–7 days (22). Furthermore, denaturation can be performed by treatment with formaldehyde vapor instead of by heating (23). These methods would have to be evaluated alone and in combination to find the best possible way to remove immunofluorescence stains.

Image analysis of the acquired images was performed in order to remove observer bias and get more quantitative data out of the performed experiments. All the steps are fully automatic except for the manual correction of errors in the segmentation. As all images of an experiment are registered to a common reference image, the segmentation is only done in a single image for a full experiment. The 3D extension of the 2D segmentation results in small errors when cells overlap. A better segmentation algorithm for 3D and correction tools for 3D segmentation are under consideration.

This study concentrated on analysis of antigens located in the cell nucleus. By staining the cytoplasm (and/or the cell membrane) and segmenting the images based on this stain, antigens located in the cytoplasm can be investigated on the single cell level. Methods for cytoplasm segmentation are currently under development.

The future development of SIFS will certainly result in better protocols for stain removal, as well as in equipment allowing highly automated image acquisition, stain removal, and staining. The higher degree of reproducibility acquired by automating the process will be a major advantage and may even allow quantitative measurements of the levels of the investigated antigens. Specialized software will probably be developed, allowing better presentation of acquired data, thus simplifying the analysis. Our vision is a system that automatically performs several rounds of SIFS followed by a classic HTX-eosin staining. The data would be presented as an HTX-eosin image with synthetic colors added to show the relative, or maybe even absolute, levels of the investigated antigens in each cell. With such a system, it would be easy to evaluate the distribution of a very large number of antigens in relationship to each other and to the morphology of the tissue. The status of complicated pathways such as ARF-p53-MDM2-p21-CDK2-pRB-E2F or signaling cascades could then be studied on the individual cell level in tissue sections or on tissue micro arrays.

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