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A Reproducible Technique for Specific Labeling of Antigens Using Preformed Fluorescent Molecular IgG-F(ab')₂ Complexes From Primary Antibodies of the Same Species

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Abstract

Immunolabeling two different antigens using the indirect approach with antibodies from the same species is not possible as secondary antibodies can bind to either primary target antibodies. In this study, we describe how preformed complexes of primary and secondary labeled antibodies can be used in such circumstances. In this situation, the first antigen is labeled using the conventional indirect method followed by incubation with the preformed primary–secondary antibody complex against the second antigen. To prevent unbound secondary antibody from binding the indirectly-labeled antibodies, resulting in a false positive, we quenched excess secondary antibody with nonimmune murine serum from the species of the primary antibody. Before the formation of the preformed complex, the optimum dilution of both primary and secondary antibodies was determined. Once these concentrations were established, the concentration of nonimmune murine serum required to quench excess unbound secondary was determined. This step was accomplished by first incubating the sample with an antibody against an antigen known to be localized away from the antigen of interest, followed by the preformed complex. If specific staining was seen, other than that expected from the preformed complex, then the concentration of the serum was deemed insufficient for quenching, and increased accordingly. We demonstrate that this approach is successful in determining the optimum conditions for the preformation of ascites and purified monoclonal primary IgG with fluorescently conjugated F(ab')₂. Double immunolabelling of two focal adhesion antigens and two cytoskeletal proteins, with two murine primary antibodies, are presented as examples of the methodology.

Keywords

double immunostaining; preformed mouse IgG-F(ab')₂ complex; colocalization

INTRODUCTION

Many research laboratories have a practical need to simultaneously localize multiple intracellular proteins by immunostaining techniques using primary antibodies raised in the same host animal, typically in the mouse. Commercially developed kits for this purpose are available (e.g. Zenon®, Invitrogen), enabling the user to create fast, versatile, and reliable antibody conjugates. Although these systems may be used for ascities or hydridoma supernatant derived antibodies, the system becomes far more experimental creating a higher

possibility for a false positive. Therefore, there is a need for a standard and reproducible methodology that can be used to simultaneously localize multiple intracellular proteins with antibodies from the same species. A reliable method would therefore permit the use of any labeled Fab secondary antibody for creating preformed complexes without the added expense and confinement of a kit system. In this case such a methodology would allow for the creation of preformed complexes with secondary antibodies conjugated to Qdots (Invitrogen) or Proximity Ligation Assay probes (Olink Biosciences), which are not currently available in kit form.

Colocalization of intracellular proteins, with primary antibodies raised in the same species, is however problematic. Direct labeling (Coons and Kaplan, 1950) of the antigen with a primary antibody conjugated to a tag is possible but not always practical due to their limited quantity. Using the two-step indirect labeling technique, in which first labeling the antigen with the primary antibody is followed by a secondary antibody against the primary antibody, must often be used to generate sufficient signal for localization (Coons et al., 1955). The tag-conjugated secondary antibody allows for visualization of the antigen-antibody complex. Some of the most common tags include fluorescent, gold, or enzyme-based conjugates. As the tag is present on the secondary antibody, which is usually plentiful and can bind to many epitopes on the primary antibody, this further amplifies the signal facilitating the detection of the antigen. By using different tags on the secondary antibody many antigens can be detected simultaneously on a single sample providing the primary antibody comes from a different species. However, in cases where both the primary antibodies have been developed in the same species the above methodology cannot be used.

Many techniques have been developed to circumvent the problem of double labeling with primary antibodies raised in the same species. These include the use of subclass specific secondary antibodies (Tidman et al., 1981); inactivating the anti species antibody with silver enhancement (Bienz et al., 1986) or by microwaves (Tornehave et al., 2000). Others have used more complex methods, such as a second biotinylated primary antibody (Wurden and Homberg, 1993) or the highly sensitive tyramide signal amplification (Shindler and Roth, 1996). A far simpler method was to conjugate two primary antibodies with their specific IgG secondary antibodies labeled with different tags (Krenacs et al., 1991). This methodology was further improved by utilizing the monovalency of the Fab fragment to saturate the entire primary antibody before adding the second primary (Nogoesu et al., 1994). However, a major concern when conjugating the primary antibody to the secondary is the existence of unconjugated secondary antibody that may produce non-specific labeling. One way of neutralizing unconjugated secondary antibody is to quench or inactivate it with nonimmune serum from the same species as the primary antibody (Hierck et al., 1994; Kroeber et al., 1998).

We present the development and testing of a novel technique that can reproducibly and reliably detect the specific colocalization of intracellular proteins by using two different primary antibodies from the same animal species using commercially available secondary antibodies commonly used for immunolocalization. This method of in situ conjugation utilizes the specificity of the Fab fragment of IgG to the secondary antibody with excess unbound Fab sites being quenched by nonimmune serum from the primary species (Hierck et al., 1994; Kroeber et al., 1998). Although in theory, the indirect labeled protein should be saturated with secondary IgG, we found that if excess secondary Fab conjugated in situ was not successfully quenched by nonimmune serum the excess secondary Fab could interact with the indirectly labeled primary antibody. The result of this interaction would result in a false positive of colocalization. To reduce the likelihood of a false positive, we designed a method to determine whether the quenching was entirely completed by the nonimmune serum.

MATERIALS AND METHODS

Cell Culture

HaCaT cells (a human keratinocyte cell line, and a gift from Dr. Hubert Fusenig, Germany Cancer Center, Heidelberg, Germany) and gingival fibroblasts (DC27) isolated from adult human gingiva as described previously (Häkkinen and Larjava, 1992) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, Irvine, UK) supplemented with 23 mM sodium bicarbonate, 20 mM Hepes (Gibco Biocult, Paisley, UK), antibiotics (50 µg/mL streptomycin sulfate, 100 U/mL penicillin), and 10% heat-inactivated fetal bovine serum (FBS, Gibco). HaCaT cells were seeded at a concentration of 10,000 cells/cm² in DMEM on bovine fibronectin (a major extracellular matrix protein) coated glass cover slips (20 µg/mL) and incubated overnight as described previously (Narani et al., 2007).

Co-culturing of Keratinocytes and Fibroblasts

Co-cultures of HaCaT cells and DC27 cells were used to demonstrate the usefulness of the labeling technique in two different cells lines cultured simultaneously. Both cell types were seeded at a concentration of 25,000 cells/cm² on bovine fibronectin-coated glass cover slips (20 µg/mL) and maintained in DMEM containing 10% FBS until confluent.

Indirect Immunofluorescence

All procedures were carried out at room temperature unless otherwise stated. Before fixation, cell culture medium was removed and the cells were rinsed with 0.1 M phosphate buffered saline (PBS) at a pH of 7.4 for 2 min. The cells were then fixed in 4% formaldehyde (Canemco Supplies, Canton de Gore, Quebec, Canada) containing 5% sucrose in PBS for 20 min followed by permeabilization by 0.5% Triton X-100 (Sigma-Aldrich, Canada, Oakville, Ontario, Canada) in PBS for 4 min. Following a rinse with PBS, the non-specific binding sites were blocked with 10 mg/mL of bovine serum albumin (BSA, Sigma-Aldrich) in PBS containing 1 mg/mL glycine (Sigma-Aldrich) for 30 min. After blocking all primary antibodies were incubated at 4°C overnight. Unbound primary antibodies were removed by rinsing (6 × 5 min) followed by incubating with a fluorescently labeled secondary antibody for 1 h (Invitrogen Canada, Burlington, Ontario, Canada). Unbound secondary antibodies were also rinsed twice in PBS then mounted with Prolong Gold Antifade solution (Invitrogen). Detailed information on the primary and secondary antibodies are given in Table 1.

Conjugating Primary Antibodies With Fluorescent Antibody in Situ

Before conjugating the secondary antibody to the primary antibody the optimal concentrations of both antibodies were determined by using the indirect method described in the previous section. Generally, those conditions were considered optimal when the primary antibody concentration was sufficient to saturate all antigenic sites in the sample of interest and that the secondary antibody concentration provided the best signal to noise ratio. The conjugation of the primary antibodies to the secondary antibody was carried out at room temperature following the steps below and is schematically represented in Figure 1:

1. The primary and secondary antibodies were mixed together in a test tube and incubated in the dark for 1 h.
2. Excess secondary antibodies not conjugated to the primary antibody were quenched by adding a predetermined concentration of IgG from murine serum (Sigma-Aldrich), which was added to the preformed complex and incubated for a further 1 h.

3. The preformed primary secondary antibody mixture was then incubated with the cells for 3 h.

Evaluating the Quenching of Unbound F(ab')₂

We used keratinocyte focal adhesion proteins migfilin (Tu et al., 2003) and paxillin (Turner et al., 1990) and a marker of early endosomes (EEA1, Mu et al., 1995) to evaluate the quenching of unbound F(ab')₂. Cells were first labeled with anti-EEA1 for 1 h. EEA1 is a hydrophilic peripheral membrane protein present in the endosome and was chosen as it is located away from the cell adhesion proteins. After rinsing for 6 × 5 min in PBS, antibodies against migfilin or paxillin conjugated to AlexaFluor 594 F(ab')₂ fragment of goat anti-mouse IgG (H+L) (Invitrogen) were quenched with IgG from murine nonimmune serum and incubated with the cells previously labeled with the EEA1 antibody. Any staining of EEA1 suggested that the quenching of the AlexaFluor 594 by the IgG from murine nonimmune serum at that concentration was not sufficient. An additional control was used to determine if the quenching step had any detrimental effect on the primary antibody. In this case the cells were labeled with mouse anti-human migfilin directly conjugated to FITC (Tu et al., 2003), at a concentration of 10 µg/mL, and compared to that of the preformed complex staining pattern.

In another set of experiments, we labeled first another antigen located in the focal adhesions of keratinocytes, namely vinculin (Geiger, 1979). We chose cytokeratin 14 as the second antigen because its distribution is distinct from focal adhesions (Purkis et al., 1990). After labeling the first antigen, the specimens were rinsed for 6 × 5 min in PBS followed by the antibody against cytokeratin 14 IgG conjugated to AlexaFluor 488 F(ab')₂ fragment of goat anti-mouse IgG (H+L) (Invitrogen) quenched with IgG from murine serum. Any staining of vinculin suggested that the quenching of the AlexaFluor 488 by the IgG from murine serum at that concentration was not sufficient.

Double Labeling Antigens with Mouse Antibodies

Double Labeling of Keratinocyte Focal Adhesion Proteins—The relatively newly discovered focal adhesion protein kindlin-1 was indirectly labeled in HaCaT cells using the indirect method with the green fluorescent AlexaFluor 488 conjugated to the F(ab')₂ fragment of goat anti-mouse antibody. We then tested whether kindlin-1 colocalizes with other focal adhesion proteins. Antibodies to migfilin and paxillin were conjugated to F(ab')₂ fragment of goat anti-mouse antibodies conjugated to the red fluorescent AlexaFluor 594. Any unbound fluorescent antibodies were quenched with IgG from murine nonimmune serum. First the cells were incubated with anti-kindlin-1 primary antibody and then incubated with F(ab')₂ fragment of goat anti-mouse antibodies conjugated to the green fluorescent AlexaFluor 488. The same cells were then incubated with the preformed complex of migfilin or paxillin antibodies and F(ab')₂ fragment of goat anti-mouse antibodies conjugated to the red fluorescent AlexaFluor 594 for an additional 3 h and processed for imaging accordingly.

Double Labeling of Antigens in Keratinocyte-Fibroblast Co-cultures—Co-cultures of keratinocytes and fibroblasts are often used to study cellular interactions and require identification of antigens in specific cell types. We chose collagen type VII as the primary antigen to study and asked whether it is expressed in one or both cell types. Type VII collagen was labeled using the indirect method (described previously) with the red fluorescent AlexaFluor 594 conjugated to the F(ab')₂ fragment of goat anti-mouse antibody. We then identified keratinocytes in these co-cultures by keratinocyte specific anti-cytokeratin 14 antibody. Anti-cytokeratin 14 was pre-conjugated with the secondary

antibody containing AlexaFluor 488 F(ab')₂ fragment, quenched and incubated with the same cells for an additional 3 h and processed for imaging described later.

Imaging

All samples were imaged under oil immersion with the x63 objective of a Zeiss Axioscope II (Carl Zeiss, Jena, Germany). The AlexaFluor 488 labeled samples were imaged using the EGFP filter and AlexaFluor 594 using the Rhodamine filter. Images were captured with a Qimaging QICam digital camera (QImaging, Surrey, British Columbia, Canada) using Northern Eclipse software (Empix Imaging, Mississauga, Ontario, Canada) and saved as 8-bit greyscale images in TIF file format. Each image was then processed for merging, which involved digitally applying a color map for each filter (green for EGFP and red for Rhodamine) onto the greyscale image. Both coloured images were then merged using image-matching software available on Northern Eclipse.

RESULTS

Indirect Labeling

Localization of kindlin-1 and paxillin, by indirect labeling in HaCaT cells showed distinct labeling in the focal adhesion sites at the periphery of the cells (Figs. 2A and 2B). Vinculin staining was present in both the focal adhesions and cell-cell contacts (not shown). Migfilin showed distinct localization in both focal and cell-to-cell adhesion sites (Fig. 2C). EEA1 was distributed in the cytoplasm and could be recognized by the distinct staining of vesicles especially around the cell nucleus (Fig. 2D). Collagen VII was present intracellularly as distinct vesicles around nuclei in keratinocytes (Fig. 2E), but staining in the fibroblasts was reticular in arrangement and of much higher intensity (not shown). Cytokeratin 14 staining was specific only to the cytoskeletal microfilaments of the HaCaT cells (Fig. 2F).

Evaluating the Quenching of Unbound F(ab')₂

The concentration of nonimmune serum required to quench excess secondary antibody of AlexaFluor 594 F(ab')₂ fragment of goat anti-mouse IgG (H+L) varied depending on the primary antibody. For anti-migfilin antibody, EEA1 staining was undetectable after adding 30 µg/mL of murine nonimmune serum to the primary-secondary antibody complex mixture (Figs. 3A–3C). In the case of anti-paxillin antibody, 20 µg/mL was sufficient to eliminate any EEA1 staining with unbound secondary antibody (Fig. 3D). Vinculin staining was observed with the preformed cytokeratin 14-AlexaFluor 488 F(ab')₂ complex with 30 µg/mL of murine nonimmune serum (Figs. 4C–E). Increasing the concentration to 40 µg/mL eliminated any vinculin staining (Fig. 4F). To eliminate any possibility that the quenching step may affect the primary antibody the cells were directly labeled with mouse anti-human migfilin conjugated to FITC and compared to the preformed complex. The labeling patterns of both complexes were identical when compared (Fig. 3C vs Fig. 3G).

Double Labeling Antigens With Mouse Antibodies

Staining intensity of paxillin or migfilin-AlexaFluor 594 F(ab')₂ complex, and cytokeratin 14 - AlexaFluor 488 F(ab')₂ complex was not as bright as that if they had been indirectly labeled. This aspect did not affect the labeling localization. A slight increase in the exposure time provided enough signal for imaging. This localization was evident if the indirectly stained paxillin (Fig. 2B) was compared to preformed complex labeling (Fig. 3E) or indirectly-labeled migfilin (Fig. 2C) was compared to the preformed complex labelling (Fig. 3F).

Double Labeling of Focal Adhesion Proteins in Keratinocytes

The double labeling of kindlin and migfilin confirmed that they indeed are co-localized in the focal adhesions (Fig. 5C). Migfilin staining was very strong in both the focal adhesions and in the cell–cell adhesions (Fig. 5B), whereas kindlin-1 was only present in the focal adhesions (Fig. 5A). Kindlin-1 also co-localized with paxillin in the focal adhesions (Figs. 5D–5F).

Collagen Type VII and Cytokeratin 14 Immunolabelling in Fibroblast-Keratinocyte Co-cultures

Fibroblasts in the confluent co-cultures surrounded the islets of HaCaT keratinocytes. Keratinocytes could be distinguished from the fibroblasts by the staining of cytokeratin, which is only present in the cytoskeleton of the epithelial cell (Fig. 5G). Collagen type VII staining was detected in both cell types but the distribution was different. The collagen type VII could be identified as intracellular cytoplasmic vesicles in the epithelial cell and seemed to concentrate in the area surrounding the nucleus (Fig. 5H). In contrast, collagen type VII staining in fibroblasts was much stronger than in the epithelial cells and in some cases had reticular distribution within the whole cell (Fig. 5H).

DISCUSSION

Localizing antigens in the same cell is important in the understanding of cellular processes. If primary antibodies are raised in different donor species, using the indirect method of labeling is not a problem. If a variety of antigens are to be identified, but only primary antibodies raised in the same species are available, then the process becomes problematic. There are many approaches to tackle this complex problem but only two known methods utilize the quenching ability of IgG from the same species as the primary antibody to bind unconjugated secondary antibody in the preformed complex mixture (Hierck et al., 1994; Kroeber et al., 1998). These hybrid methods of indirect and direct immunocytochemistry involve non-covalent labeling of the primary with a covalently labeled secondary antibody. The first step of the double labeling process is to indirectly label the sample with the first primary antibody followed by the secondary antibody conjugated to a fluorescent label. The second step involves coupling the second primary IgG with the secondary IgG conjugated to a fluorescent label with a different emission spectrum to the first, in a reaction tube. Any uncoupled secondary antibody is then removed from the system by the addition of nonimmune serum, which quenches the free binding site of the secondary IgG. The preformed immune complex is then applied to the indirectly labeled sample. By using this system, the secondary fluorescently labeled IgG in the preformed immune complex cannot interact with the first IgG because it is blocked by nonimmune serum. As a result, it is possible to successfully co-localize two antigens in the same cell with primary antibodies from the same species. One significant, previously unrecognized, problem with this technique is the high probability of a false positive for colocalization. Unless the exact concentration of nonimmune serum required to completely quench any uncoupled $F(ab')_2$ has been determined, unbound $F(ab')_2$ can bind to any free primary antibody from the indirectly labeled antigen. Thus, the goal of this study was to develop a complete and reproducible method with rigorous controls that would rule out any concern for non-specific labeling when two antigens, localized in the same cell, are detected with primary antibodies from the same species.

We were successful in developing a reproducible and specific method to co-localize two antigens in the same cell with primary antibodies from the same species using secondary antibodies that are commonly used in immunolocalization. By applying this method investigators will have a greater choice of secondary antibodies available to them and

therefore are not confined to buying expensive commercial kits. Our method in this investigation differed from that of Kroeber et al. (1998) in that all of the secondary antibodies were $F(ab')_2$ fragments covalently linked to a fluorescent probe rather than an IgG. Using $F(ab')_2$ fragments as secondary antibodies are advantageous in that they do not disrupt the structure of the primary IgG antigenic site. In addition they increase the probability of specific binding to the primary antibody because the eliminated Fc portion of the IgG may interact with Fc receptors on the cells. However, the fluorescence intensity of preformed molecular complexes was found to be lower than those samples labeled using the indirect method. Because $F(ab')_2$ fragments were used the likelihood of the secondary antibody changing the affinity of the primary antibody is minimal. An explanation for the decrease in fluorescence intensity is that the signal to noise ratio of labeled antigen is increased as a result of the quenching step of non-specific unbound $F(ab')_2$ (Hierck et al., 1994). One way to increase the intensity of fluorescence would be to use nanocrystal Qdots (Giepmans et al., 2005). Qdots have greater stability than the organic fluorochromes, have a broad adsorption spectrum and fluoresce at narrow but symmetric emission spectra. The option of using Qdots in this system is currently under investigation.

Non-specific binding of the antibody with the sample should be minimal to be able to conclude that the antigen of interest has been detected specifically. Invariably, in this situation, where both primary antibodies are from the same species, the conjugated secondary antibodies from the preformed complex could bind specifically to the first primary IgG that was indirectly labeled before the addition of the preformed complex. Pre-determining the success of the complex pre-formation in the reaction tube, in addition to the efficiency of the secondary free-binding site quenching, is critical to the specificity of the double labeling. Firstly, to increase the chances of specific binding, bivalent $F(ab')_2$ secondary antibodies were used rather than IgG. Secondly, we devised a test to determine the ideal concentration of $F(ab')_2$ and the concentration of nonimmune serum required to completely quench any uncoupled $F(ab')_2$. In this way the preformed complex would only label the second antigen without any unbound $F(ab')_2$ binding to any free primary antibody from the indirectly labeled antigen. By following this general methodology the technique could be modified accordingly to successfully double label antigens with primary antibodies from the same species in a reproducible manner in any labeling system. A summary of the stages necessary for successful double labeling are depicted schematically in Figure 1. Appropriate controls are crucial in the critical evaluation of the result. Therefore an example of the experimental layout can be seen in Figure 6.

In conclusion, this report offers a reliable method to specifically co-localize two antigens in the same cell with primary antibodies from the same species using secondary antibodies that are commonly used for immunolocalization minimizing the likelihood of a false positive. The result of using such a method allows specific labeling of two antigens with primary antibodies from the same species with high signal-to-noise ratio. Multiple labelings of more than two antigens could be possible, however, this would depend on the affinities of antibodies used.

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Fig. 1.

A schematic representation of the methodology for generating preformed molecular complexes and the testing of whether excess unbound secondary F(ab')₂ conjugated to the fluorescent label has been successfully quenched. **A:** The first antigen (in this case kindlin-1) is labeled indirectly by a primary IgG followed by the secondary F(ab')₂. **B:** The preformed complex against the second antigen (migfilin in this case) is generated by mixing the primary IgG with the secondary F(ab')₂ conjugated to the fluorescent probe. To eliminate any non-specific labeling of the first indirectly labeled antibody by unbound secondary of the preformed complex a pre-determined amount of normal nonimmune serum from the primary antibody species is added to quench excess secondary F(ab')₂. **C:** The successful quenching of excess F(ab')₂ is determined by first labeling the sample with only a primary IgG against another antigen located in an area that is located far from the one of interest (EEA1 is used as an example in this case). The preformed complex (migfilin IgG-F(ab')₂) is then added. If the concentration of the nonimmune serum is insufficient for quenching, excess indirect labeling of the primary IgG will be seen in addition to that of the preformed complex antigen. If the secondary F(ab')₂ is successfully quenched, then only labeling of the preformed complex antigen will be seen. **D:** The preformed complex can then be applied to specifically label a second antigen on the sample without interfering with the first indirectly labeled antigen even though the primary antibodies are from the same

species. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 2. HaCaT cell islets indirectly labeled with mouse anti-human primary IgG and located with goat anti-mouse F(ab')₂ covalently linked to AlexaFluor 488 or AlexaFluor 594. Fluorescence is located in distinct areas at the periphery of the cells (arrows) when primary IgG against kindlin-1 (**A**) paxillin (**B**) and migfilin (**C**) are used. Migfilin is also visible in cell-to-cell contacts between the cells (grey arrow). EEA1 (**D**) and collagen type VII (**E**) can be seen in the cytoplasm as distinctive vesicular structures that are more numerous around the nuclei (N) (arrows). Cytokeratin 14 is visible as a cytoskeletal structure distributed throughout the whole cell (**F**). Scale bar 10 μ m.

Fig. 3.

Images displaying the evaluation that the preformed complex (mouse anti-human migfilin IgG- goat anti-mouse F(ab')₂ AlexaFluor 594) did not contain any unbound secondary antibody in the mixture. Mouse anti-human EEA1 was incubated with HaCaT cells followed by the preformed complex. Excess unbound secondary antibody was quenched with murine nonimmune serum after the primary-secondary antibody complex was formed. The distinctive vesicular structures (black arrow) in EEA-1 labeled cells are evident (**A**) however at low concentrations (20 µg/mL) of murine nonimmune serum, migfilin staining in addition to EEA1 staining (arrow) is visible (**B**). Increasing the concentration of murine nonimmune serum (30 µg/mL) eliminated any EEA1 staining (**C**). A lower concentration of mouse serum (20 µg/mL) was required for the successful quenching of excess secondary antibody with anti-paxillin antibody (**D**) (arrow). When preformed complexes are used to label paxillin (**E**) and migfilin (**F**) (arrows), respectively, the signal to noise is much higher although the intensity of the fluorescence is not as bright as indirectly labeled antigens (compare to Figs. 2B and 2C). Directly labeling the cells, with mouse anti-human migfilin conjugated to FITC shows identical labeling patterns (arrow) to that of the preformed complex (compare Figs. 3G–3F). Scale bar 10 µm.

Fig. 4.

Images displaying the evaluation that the preformed complex (mouse anti-human cytokeratin 14 IgG- goat anti-mouse F(ab')₂ AlexaFluor 488) did not contain any unbound secondary antibody in the mixture. Images **A** & **B** show examples of the indirect labeling of vinculin (arrow) and cytokeratin 14 (arrow), respectively, in co-cultures of HaCaT epithelial cells (k) and DC27 fibroblasts (f). Vinculin is present in focal and cell-cell adhesions in both cell types, whereas cytokeratin 14 is specific only to the HaCaT cells. When anti-human vinculin antibody incubation was followed by the preformed complex, and excess unbound secondary antibody was quenched with nonimmune murine serum at low concentration (20 µg/mL), cytokeratin 14 staining is visible (arrow) but vinculin staining is not (**C**). At a higher magnification the staining of vinculin is clearly seen on the periphery of the fibroblast (arrow) (**D**). Increasing the concentration of nonimmune murine serum (30 µg/mL) decreased any vinculin staining (**E**). A concentration of 40 µg/mL nonimmune murine serum was required for the successful quenching of excess secondary antibody (**F**).

Fig. 5.

Images displaying the successful double labeling of anti-gens with antibodies from the same species. Indirect labeling of kindlin-1, with mouse anti-human kindlin-1 IgG with goat and mouse F(ab')₂ AlexaFluor 488, is concentrated in the focal adhesions (arrow) of the HaCaT cell (A). When the preformed complex of mouse anti-human migfilin-goat anti-mouse F(ab')₂ AlexaFluor 594 is applied to the same cell (arrow) the staining is also present in the focal adhesions (B). When both images are merged both antigens are co-localized (arrow) in the focal adhesions (C). By following the same methodology the indirectly labeled kindlin-1 (D) is present at the periphery of the cell (arrow) and the pre-formed complex of mouse anti-human paxillin-goat anti-mouse F(ab')₂ AlexaFluor 594 (E) follows the same pattern. Merging both images shows that both proteins are colocalized (F). In co-cultures of HaCaT epithelial cells (k) and DC27 fibroblasts (f), the preformed complex of anti-cytokeratin 14 specifically labels a cytoskeletal structure (arrow) that is distributed throughout the whole epithelial cell (G). The indirectly labeled collagen type VII can be seen in the cytoplasm of the epithelial cells as distinctive vesicular structures (asterisk in H) that are more numerous around the nuclei (N). Collagen type VII is more concentrated in the fibroblasts and in some cases has a reticular arrangement (arrow) in the cell cytoplasm (H). When both images are merged, the epithelial cells can be distinctively identified from the surrounding fibroblasts by the keratin cytoskeleton staining. Collagen VII is present in both cells types but organized in a different manner (I). Scale bar 10 μm.

1° = mouse anti-kindlin IgG 2° = goat anti mouse Alexa 488 F(ab') ₂	1° + 2° = preformed anti-migfilin IgG coupled with goat anti mouse Alexa 594 F(ab') ₂	1° = mouse anti kindlin IgG + 2° Alexa 488 F(ab') ₂ + 1° + 2° = preformed mouse anti-migfilin IgG coupled with Alexa 594 F(ab') ₂
Goat anti mouse Alexa 488 F(ab') ₂ secondary control	Goat anti mouse Alexa 594 F(ab') ₂	Goat anti mouse Alexa 488 F(ab') ₂ + Goat anti mouse Alexa 594 F(ab') ₂

a

1° = mouse anti-EEA1 IgG 2° = goat anti mouse Alexa 488 F(ab') ₂	1° = preformed mouse anti-migfilin IgG coupled with secondary Goat anti mouse Alexa 594 F(ab') ₂ + mouse serum
Goat anti mouse Alexa 488 F(ab') ₂ secondary control	1° = mouse anti-EEA1 IgG + 1° + 2° = mouse anti-EEA1 IgG + preformed mouse anti-migfilin IgG coupled with secondary Alexa F(ab') ₂ 594 + mouse serum

b

Fig. 6. Schematic representation of the different control variants used to successfully label two antigens on the same sample with two primary antibodies that are from the same species. **(a)** Control variants to eliminate any non-specific labeling from the secondary F(ab')₂. **(b)** Control variants to generate preformed complexes with successful quenching of excess F(ab')₂.

TABLE 1

A detailed inventory for each primary antibody used in this study including concentrations and supporting documentation. Also noted are the concentrations and specifics for each corresponding secondary antibody, used in this study

Primary antibody	Source	Concentration	Secondary antibody	Concentration
Mouse anti-human kindlin-1	Cell culture hybridoma produced in our laboratory Papachristou et al., 2007.	Undiluted	Goat anti-mouse IgG (H+L) AlexaFluor 488 F(ab') ₂	5 µg/mL
Mouse anti-human collagen type VII	Monoclonal-Clone LH7.2; Chemicon International Inc., TemelUCA, CA	2.5 µg/mL	Goat anti-mouse IgG (H+L) AlexaFluor 488 F(ab') ₂	2.5 µg/mL
Mouse anti-human paxillin	Monoclonal-Clone 349; BD Transduction Laboratories, Mississauga, Ontario, Canada	25 µg/mL	Goat anti-mouse IgG (H+L) AlexaFluor 594 F(ab') ₂	0.25 µg/mL
Mouse anti-human migfilin	Mouse ascities fluid produced in our laboratory Tu et al., 2003.	0.25 µg/mL	Goat anti-mouse IgG (H+L) AlexaFluor 594 F(ab') ₂	5 µg/mL
Mouse anti-human cytokeratin 14	Monoclonal-Clone LL002; Cedarlane Laboratories Ltd., Hornby, Ontario, Canada.	10 µg/mL	Goat anti-mouse IgG (H+L) AlexaFluor 488 F(ab') ₂	10 µg/mL
Mouse anti-human early endosome antigen	Monoclonal-clone 14; BD transduction laboratories, Canada.	25 µg/mL	Goat anti-mouse IgG (H+L) AlexaFluor 488 F(ab') ₂	2.5 µg/mL
Mouse anti-human vinculin	Monoclonal-clone h VIN-I; Sigma	2.4 µg/mL	N/A	N/A