Ki-67 Membranous Staining: Biologically Relevant or an Artifact of Multiplexed Immunofluorescent Staining

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Abstract: In the process of developing a multiplex of 8 common breast cancer biomarkers (Her2/neu, estrogen receptor, progesterone receptor, Ki-67, aldehyde dehydrogenase-1, Na^+K^+-ATPase, cytokeratin 8/18, and myosin smooth muscle) on a single formalin-fixed paraffin-embedded slide using a sequential staining, imaging, and dye bleaching technology developed by General Electric Company, membranous Ki-67 staining was observed and colocalized with Her2/neu staining. Using immunohistochemistry as gold standards, we discovered that membranous Ki-67 was an artifact caused by the binding of cyanine 5-conjugated rabbit polyclonal Ki-67 antibody to a secondary cyanine 3-conjugated donkey anti-rabbit antibody which was previously applied and bound to rabbit Her2/neu antibody in our multiplexing experiment. After blocking with rabbit serum, a successful protocol for 8 biomarker multiplexing without cross-reactivity of antibodies from the same species was developed.

Key Words: multiplexing, immunofluorescence, Ki-67, Her2/neu, artifact, cross-activity

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Immunofluorescence (IF)-labeling techniques combined with digital image processing present exciting opportunities for depicting the colocalization of multiple proteins on a single formalin-fixed paraffin-embedded tissue section. This information is potentially valuable in elucidation of the molecular interactions involved in complex signal transduction pathways. However, conventional IF techniques are often limited to 2 to 4 of biomarkers at 1 time due to the spectral overlap of fluorescent dyes. To overcome these limitations, multiepitope ligand cartography used photo-bleaching to create topological maps of hundreds of proteins.[1,2] At General Electric Company, a novel multiplexed fluorescence microscopy method (MxIF) employing chemical inactivation of fluorescent dyes after imaging has been developed,[3] and is currently commercialized as MultiOmyx at Clarient (a GE HealthCare Company). This allows the same fluorophores to be reused on different dye-conjugated primary antibodies in an iterative sequence of staining, imaging, and signal inactivation. This overcomes the limitation due to spectral overlap in conventional multimarker IF. In addition, the use of direct antibody-fluorophore conjugation in principle eliminates the need for secondary antibodies, and, therefore, removes technical constraints due to species interactivity. Using MxIF, up to 61 biomarkers associated with colorectal cancer have been multiplexed and imaged on tissue microarrays (TMAs) with 747 colorectal cancer patients.[3] However, not all antibodies are compatible with the conjugation process; sometimes primary antibodies are still required in MxIF.

At Sunnybrook Research Institute, a prototype of an automated microfluidic device performing MxIF was tested on a set of breast carcinomas with 105 patients.[4] In this pilot study, pathologists observed concordance between MxIF and conventional immunohistochemistry (IHC) for most common breast biomarkers such as Her2/neu, estrogen receptor (ER), progesterone receptor (PgR), cytokeratin 8/18, except for Ki-67, where mysterious membranous staining was observed.

Human nuclear protein Ki-67 is associated with the DNA synthesis stage of the cell cycle and is widely used in pathology as a marker to measure the fraction of cells undergoing proliferation in human breast tumors. During the S phase of the cycling cell, Ki-67 is almost exclusively located in the nucleoli of the cell.[5] However, recently, aberrant membranous and cytoplasmic distributions of Ki-67 have also been reported in various rare tumors, including invasive breast carcinoma,[6] invasive amelanotic melanoma,[7] and sclerosing hemangioma of the lung.[8] Collectively, these lines of evidence provided a plausible basis for membranous staining observed in our hands. In this work, our aim was to investigate whether the membrane Ki-67 staining is a true biological phenomenon, as claimed in other studies, or if it is an artifact created during the staining process.

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MATERIALS AND METHODS

Antibodies and Direct Fluorophore Conjugation

For IF studies, rabbit polyclonal Ki-67 antibody (RB-1510-P1ABX) and rabbit monoclonal Her2/neu antibody (MA5-14509, clone SP3) were obtained from Thermo Fisher (Waltham, MA). Ki-67 was later directly conjugated to cyanine 59 (Cy5) using NHS-ester coupling mechanism with a dye to protein (D/P) ratio of 3.1. Mouse monoclonal ER (clone 6F11) was obtained from Leica (Buffalo Grove, IL). Secondary antibodies, cyanine 3 (Cy3)-donkey anti-rabbit IgG (711-165-152), and Cy5-donkey anti-mouse IgG (715-177-003) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Another rabbit monoclonal Her2/neu antibody (29D8) from Cell Signaling (Danvers, MA) was directly conjugated to Cy3 with a D/P ratio of 4.6. Mouse monoclonal PgR (clone PgR 1294) was obtained from Dako (Carpinteria, CA), and was conjugated to Cy5 with a D/P ratio of 4.8.

For IHC staining, we used rabbit monoclonal Her2/neu antibody (clone SP3) and rabbit monoclonal Ki-67 antibody (clone SP6) from Thermo Fisher. These 2 antibodies were approved for use in clinical pathology laboratories by the American Society of Clinical Oncology. All other reagents were obtained from Sigma-Aldrich (St Louis, MO), unless otherwise specified.

Tissues and Slide Preparation

A TMA block comprising 136 cores representing 50 cases of invasive breast cancer was obtained from the Department of Anatomic Pathology at Sunnybrook Health Sciences Centre (Research Ethics Board Approval # 338-2012). The TMA was constructed from consecutive non-selected cases accessioned in 2008 to 2009. A block from a Her2/neu-positive invasive ductal carcinoma was also obtained for use in the study because of the limited availability of TMA sections. Four-micron-thick sections were cut, dewaxed in xylene, and rehydrated in graded ethanol and distilled water. Endogenous peroxidase activity of the tissue was blocked by incubation of 3% hydrogen peroxide for 10 minutes. The subsequent staining protocols for these slides are listed in Table 1 and described below.

Eight Biomarkers Sequential Multiplexing Using an Automated Microfluidic Technology (Slide A)

Slide A was multiplexed with 8 biomarkers (ER, PgR, Ki-67, Her2/Neu, myosin smooth muscle, aldehyde dehydrogenase-1, CK8/18, NaKATPase) using automated sequential staining, imaging, and bleaching. Experimental details were published previously.[4]

Single IHC Staining of Ki-67 (Slide B) and Her2/neu (Slide C)

Antigen retrieval processing of tissue sections was done using a pressurized cooking chamber (Decloaking Chamber Plus; Biocare Medical, Concord, CA) in 0.01 M citrate buffer at pH 6.0 for 4 minutes at 110°C. Ki-67 antibody (clone SP6, 1:200) and Her2/neu antibody (clone SP3; 1:100) were incubated for 60 minutes at room temperature on slide B and slide C, respectively, then detected by Mach3 kit (rabbit probe and HRP polymer; BioCare Medical) for 30 minutes to reveal the binding of primary antibody by peroxidase staining. The substrate 3, 3-diaminobenzidine (DAB) (K3468; Dako, Glostrup, Denmark) was used to develop a brown chromogen. Finally, the sections were counterstained with hematoxylin, dehydrated, cleared, and mounted for examination.

Single Ki-67 IF Staining on TMA (Slide D) and Tissue Section (Slide E), MxIF Staining of Her2/neu and Ki-67 (Slides F to I)

All 6 slides were processed with heating to 110°C in citrate buffer (pH 6.0), and then transferred to Tris EDTA buffer (pH 9.0) for antigen retrieval. The slides were washed with phosphate-buffered saline and counterstained with 4′6′-diamidino-2-phenylindole (DAPI) (D3571; Invitrogen, Carlsbad, CA) for nuclear identification. Single Ki-67 IF staining was performed for slide D (TMA) and slide E (whole section) with direct conjugated Ki-67-Cy5 (10 μg/mL) applied to the slides for 60 minutes at room temperature. The slides were then digitized using a commercial whole slide scanner (Mirax Scan; Carl Zeiss, Gottingen, Germany) using standard Cy5 and DAPI filters.

Multiplexed staining of Her2/neu and Ki-67 were manually performed using sequential labeling of biomarkers with dye deactivation. Cy3-conjugated anti-Her2/neu antibody (clone 29D8, working concentration 5 μg/mL) was applied to slide F for 60 minutes. Primary anti-Her2/neu antibody (1:100) was applied to slide G, slide H, and slide I for 60 minutes. Secondary Cy3-conjugated anti-rabbit antibody (1:250 for 60 min) was applied to slides G and I, but was not used in slide H. Her2/neu images were acquired for slides F to I, except for slide H, as there was no Cy3 probe. Slides were then bleached with inactivation solution to remove all signals but DAPI. Normal rabbit serum (10%) was incubated with slide I, and then Cy5-conjugated anti-Ki-67 was incubated on slides F through I. All IF-labeled slides were scanned again using a Mirax scanner, with the exception of slide G images which were scanned using a TISSUEscope 4000 Scanner (Huron Technologies, Waterloo, ON, Canada).

RESULTS

Localization of Ki-67 on Breast Cancer TMA From IF or IHC Analyses

Both normal nuclear staining and membranous staining of Ki-67 were observed on slide A in which a total of 8 biomarkers were applied in the MxIF experiment (Fig. 1D). Routine single-antibody IHC staining with anti-Ki-67 (slide B) and anti-Her2/neu (slide C) antibodies applied on serial sections of the same TMA demonstrated the expected nuclear staining patterns for Ki-67 (Fig. 1E), and membranous staining for Her2/neu (Fig. 1F). All of the 136 cores on the TMA showed positive staining of Ki-67 at different levels, exclusively in

TABLE 1. Preparation of Slides A to I

Slide ID	Slide Type	Staining Condition	Platform	Her2 Antibody	Detection of Her2	Normal Rabbit Serum	Ki-67 Antibody Rabbit Polyclonal	Detection of Ki-67	Ki-67 Cellular Localization
A	TMA	Automated/multiplex with 8 biomarkers	IF	SP3 clone 1°	2° Cy3 antibody	–	+	DC with Cy5	Membrane/nuclear
B	TMA	Manual /single stain	IHC	–	–	–	+*	DAB	Nuclear
C	TMA	Manual /single stain	IHC	SP3 clone 1°	DAB	–	–	–	NA
D	TMA	Manual /single stain	IF	–	–	–		DC with Cy5	Nuclear
E	Whole tissue section	Manual /single stain	IF	–	–	–	+	DC with Cy5	Nuclear
F	Whole tissue section	Manual/multiplex (2)	IF	29D8 clone	DC with Cy3	–	+	DC with Cy5	Nuclear
G	Whole tissue section	Manual/multiplex (2)	IF	SP3 clone 1°	2° Cy3 antibody	–	+	DC with Cy5	Membrane/nuclear
H	Whole tissue section	Manual/multiplex (2)	IF	SP3 clone 1°	–	–	+	DC with Cy5	Nuclear
I	Whole tissue section	Manual/multiplex (2)	IF	SP3 clone 1°	2° Cy3 antibody	+	+	DC with Cy5	Nuclear

*Rabbit monoclonal SP6 antibody was used for this slide.
– indicates not added to this step; +, added to this step; 1°, primary; 2°, secondary; Cy3, cyanine 3; Cy5, cyanine 5; DAB, 3, 3-diaminobenzidine; DC, direct conjugates; IF, immunofluorescence; IHC, immunohistochemistry; TMA, tissue microarray.

the cell nuclei using IHC analysis. In addition, when another serial section (slide D) was stained with Ki-67 direct conjugate, no membranous staining pattern was observed across all the cores (images not shown). Results from this set of experiments on sequential TMA sections ruled out the possibility that the membranous staining was due to a biological phenomenon or issue with direct conjugates, suggesting it was likely an artifact.

IF Staining Pattern of Ki-67 on a Whole Tissue Section of Breast Cancer

To further investigate the root cause of Ki-67 artificial membranous staining, serial sections of a whole tissue sample from a Her2/neu-positive patient were used. When Cy5-conjugated anti-Ki-67 antibody was applied to a breast cancer section (slide E), we observed a nuclear staining pattern (image not shown). However, when primary rabbit anti-Her2/neu antibody was applied to a breast cancer tissue section, followed by using donkey anti-rabbit Cy3-conjugated secondary antibody, bleaching, and finally labeling with Ki-67 Cy5-direct conjugate (slide G), membranous as well as nuclear staining patterns appeared (Fig. 2C), similar to what we observed with the automated multiplexing technology (Fig. 1D). This result ruled out the use of the automated platform as a potential cause of artifacts. Figure 2A shows membranous staining of Her2/neu, and Figure 2B shows the bleached image with no Cy3 signal after chemical inactivation. When a direct Cy3-conjugated Her2/neu and a direct Cy5-conjugated Ki-67 were applied to slide F, we observed only normal nuclear staining of Ki-67 (Fig. 2F). This observation suggests that the combination of using the primary antibody for Her2/neu with secondary antibody detection led to the anomalous Ki-67staining. On slide H, we applied anti-Her2/neu antibody, without secondary antibody, and then stained the slide with anti-Ki-67 direct conjugate, resulting in nuclear Ki-67 labeling (image not shown). This confirmed the secondary antibody as the source contributing to the observed nonspecific staining. To further validate this, we incubated the slide with normal rabbit serum as a blocking step after application of Her2/neu primary antibody and secondary detection, and before the application of anti-Ki-67 (slide I). Under these conditions, only nuclear staining of Ki-67 was observed (Fig. 2I). This provides a method to prevent unwanted nonspecific Ki-67 staining.

DISCUSSION

In this study, we have demonstrated that a rabbit Cy5-conjugated anti-Ki-67 antibody cross-reacts with Cy3-conjugated secondary anti-rabbit antibody that was administered in the previous round of Her2/neu staining. This phenomenon is illustrated in Figure 3. In the MxIF experiments (slide A and slide G), donkey anti-rabbit Cy3-conjugated secondary antibody (red Y symbol in Fig. 3) was used to detect rabbit anti-Her2/neu antibodies bound to the antigen. However, not all binding sites on the secondary antibodies were occupied by anti-Her2/neu primary antibodies. Consequently, the Cy5-direct conjugated rabbit anti-Ki-67 antibody bound to the remaining sites of donkey anti-rabbit antibody (red Y) that were already bound to anti-Her2/neu primary antibodies. This explains why observed artificial Ki-67 membranous staining highly colocalized with Her2/neu staining. In Her2/neu-negative breast cancer cases, no membranous Ki-67 was observed on TMA slides. The use of normal rabbit serum as blocking in slide I is encouraging, as it shows an effective way to prevent this cross-reaction. In future

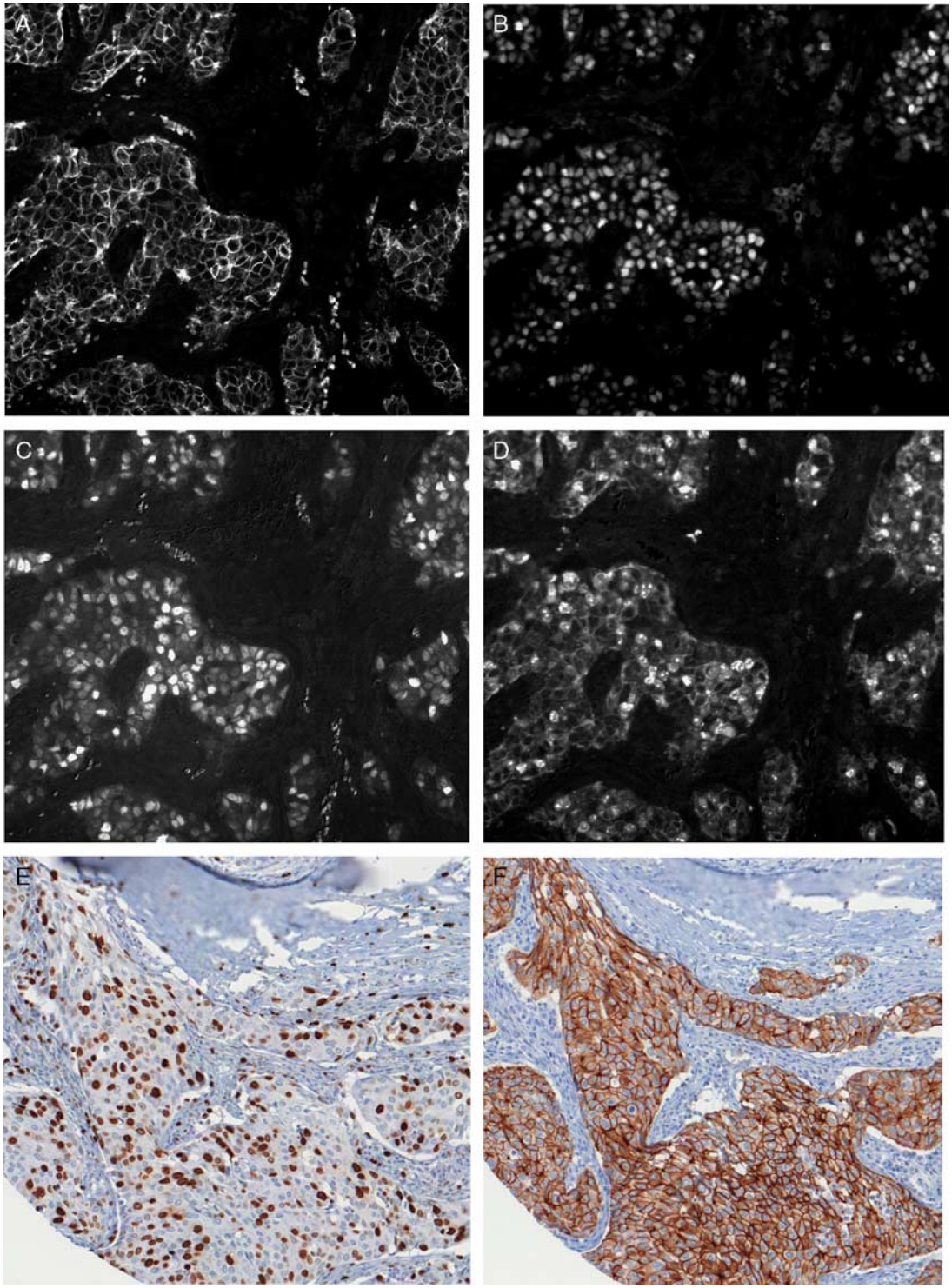

FIGURE 1. Sequential staining of Her2/neu (first round) and Ki-67 (second round) in a multiplexing experiment (slide A) and its immunohistochemistry (IHC) staining using serial sections. Top row shows normal membranous Her2/neu staining (A) and nuclear estrogen receptor staining (B) at the first round. The middle row shows nuclear progesterone receptor (C) staining and membranous/nuclear staining of Ki-67-Cy5 (D) in the second staining round. The bottom row shows IHC images of Ki-67 (slide B) (E) and Her2/neu (slide C) (F) staining using serial sections of slide A.

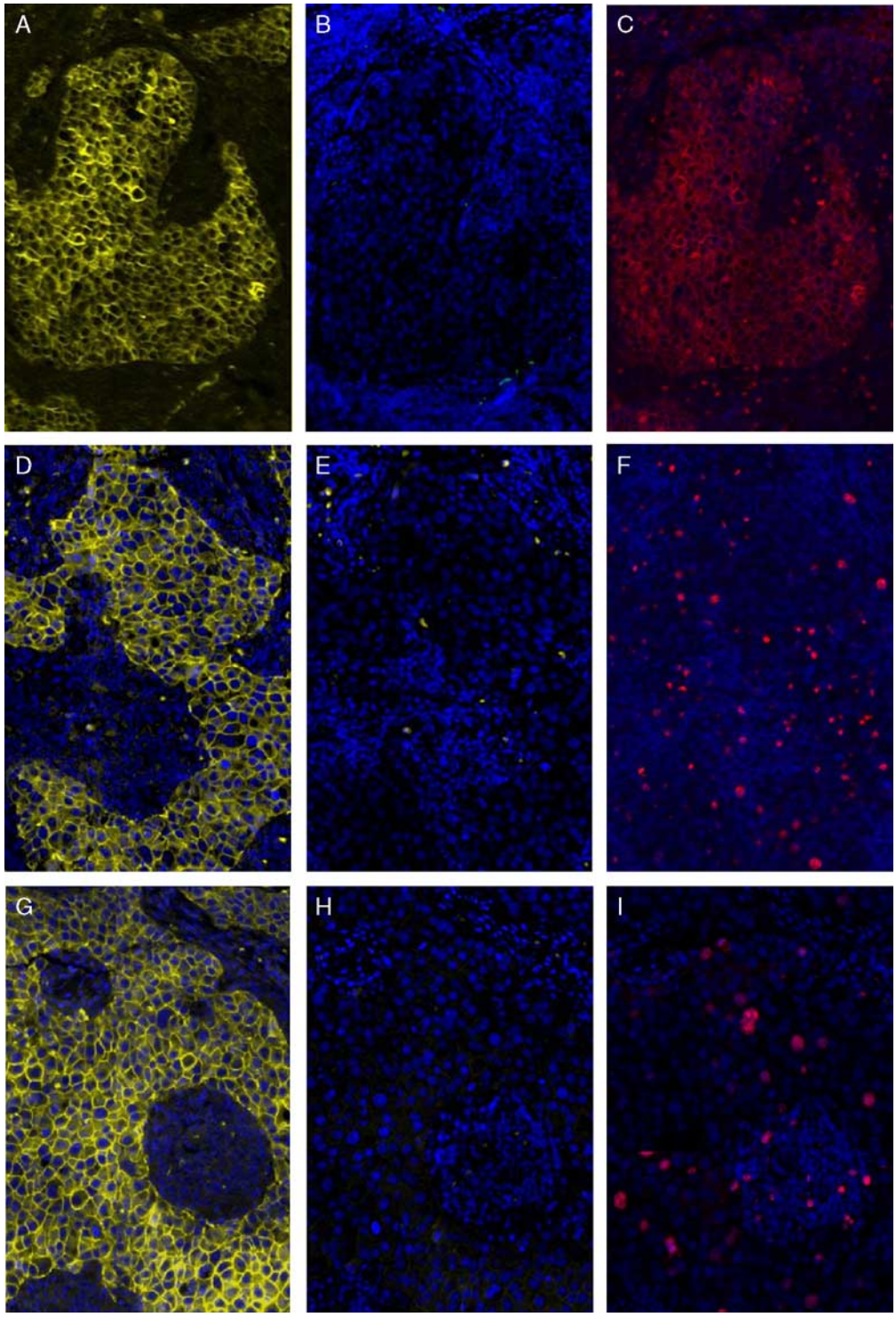

FIGURE 2. Immunofluorescence images of Her2/neu (left column), bleached images of DAPI (middle column), and Ki-67 (right column) using breast tumor sections (slides F, G, and I). Top row: representative images of double staining with unconjugated Her2/neu and conjugated Ki-67-Cy5 of a breast tumor section from slide G are shown. Both membranous and nuclear staining of Ki-67 were detected (C); middle row: representative images from slide 5 using direct conjugated Her2/neu followed by Ki-67-Cy5 staining showed nuclear staining exclusively (F); bottom row: images taken from slide 8 with staining of Her2/neu antibody and the addition of a blocking step with normal rabbit serum before staining with Ki-67-Cy5, Ki-67-positive activities returns at the nuclear compartment (I).

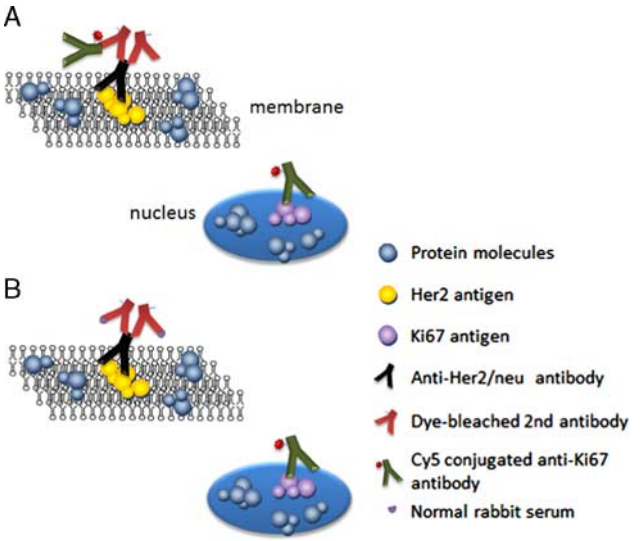

FIGURE 3. Schematic diagram illustrating the steps leading to artificial Ki-67 membranous staining. A, Without the blocking step using normal rabbit serum, direct conjugated Ki-67 (rabbit polyclonal) binds to secondary donkey anti-rabbit Cy3 already bound to Her2 antigen. B, After blocking with normal rabbit serum, these binding sites on secondary donkey anti-rabbit are no longer available and prevented artificial membranous staining.

experiments, normal rabbit serum should be applied to block any remaining binding sites on the secondary donkey anti-rabbit antibodies, or we should use direct conjugates all the time. Similarly, the application of mouse ER and mouse PgR antibodies in a sequential manner could also lead to nonspecific staining of PgR on ER-positive cells.

Although there are a number of reports that describe staining of Ki-67 on cell membranes, it is worth noting that all these unusual distribution patterns of Ki-67 are associated with the MIB-1 antibody, which is a monoclonal antibody raised against a recombinant version of the Ki-67 antigen. In our case, we used a rabbit polyclonal antibody and membranous staining was due to binding of rabbit Ki-67 antibody to remaining binding sites of secondary Cy3-conjugated donkey anti-rabbit antibody which were already bound to Her2/neu antibody. Although there is no direct comparison of the staining pattern of MIB-1 and SP6 of breast carcinoma reported in the literature, it has been shown that nuclear staining of Ki-67 by MIB-1 of breast carcinoma was highly correlated with another Ki-67 labeling antibody BGX.[9] According to current recommendations, cytoplasmic/membranous staining of MIB-1 in breast carcinoma should be ignored while creating a Ki-67 score,[10] whereas others have reported that this staining may be associated with HER2 and ER status.[6] The functional significance of membranous/cytoplasmic staining of Ki-67 demonstrated by MIB-1 thus warrants further investigation.

Multiplexing technology is a powerful tool. However, extra caution should be practiced when conducting MxIF experiments to avoid artificial staining due to species cross-reaction between antibodies. To avoid false-positive signals, a blocking step is necessary, or else one should employ only direct conjugated antibodies by eliminating the use of secondary antibody.

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