

hybridization (CISH) for HER2 on a single sample. Am J Clin Pathol 2012; 137:102-110; doi: 10.1309/AJCPLNHINN9O6YSF. While the authors were able to obtain signal by first imaging CISH sample, followed by imaging IHC sample, they found that better images were obtained when not only IHC is performed after CISH, also the sample was only imaged until the completion of all wet lab experimentation (i.e. without an intermediate imaging step). Importantly, Reisenbichler et al. could not obtain CISH signal when they first performed IHC on the sample, then performed CISH.

Gaiser et al. described a method for the analysis of tissue structures localized by protein expression for gene amplification within the same tissue sample. The authors first detected CD133 expression in colon cancer tissue samples, using monoclonal antibody and FITC-labeled secondary antibody. Using 40x magnification, regions of interest were manually selected and recorded. After imaging, FISH analysis was performed simultaneously for amplified cMyc oncogene and ZNF217. The sample was again imaged in the same regions at 40 x magnification. An automated microscope and custom designed classifier for tile based FISH analysis was used to analyze the images. Anal Cell Pathol (Amst). 2010; 33(2): 105–112; doi: [10.3233/ACP-CLO-2010-0532](https://doi.org/10.3233/ACP-CLO-2010-0532)).

There is still a need for improved methods for the detection of multiple targets in the same biological sample and the creation of a composite image to compare expression on a cell by cell basis, allowing the inclusion or exclusion of cells based on protein expression, apoptosis etc.

Summary of the Invention

In a first aspect of the disclosure, it is provided a method of generating an image of a region of interest in a biological sample comprising the steps of:

- 1) generating a first image including said region of interest of said biological sample having undergone a first protocol but not a second protocol; and
- 2) generating a second image including said region of interest of said biological sample after having undergone a second protocol;
wherein said region of interest is smaller than said sample and wherein the second protocol includes immunofluorescence detection of a target protein or fluorescence

detection of at least one target nucleic acid sequence by hybridizing a probe with the target nucleic acid sequence.

In one ~~aspect embodiment of the invention~~disclosure, it is provided a method for the generation of an image of a region of interest in a biological sample comprising the steps of (1) generating a first image including the region of interest of the biological sample having undergone a first protocol but not a second protocol; and (2) generating a second image including the region of interest of the biological sample after having undergone a second protocol; wherein the region of interest is smaller than the sample. In some embodiments, the region of interest is selected by comparing the first image of the biological sample to pre-determined criterion. In certain embodiments, the first protocol includes immunofluorescence detection of a target protein. In certain embodiments, the second protocol includes fluorescence detection of at least one target nucleic acid sequence

by hybridizing a probe with the target nucleic acid sequence. In other embodiments, the second protocol includes immunofluorescence detection of a different target protein. In certain embodiments, the method further comprises generating a composite image that includes at least the regions of interest from each of the first and the second images. In some embodiments, the composite image is generated by a method which comprises registering locations of selected signals obtained during the generation of the first image with locations of selected signals obtained during the generation of the second image. In certain embodiments, the locations of selected signals are from within the region of interest.

In certain embodiments, the first or the second protocol comprises staining the sample with a fluorescent marker that provides morphological information.

In certain embodiments, the first or the second protocol comprises detecting autofluorescence of the biological sample.

In certain embodiments, the first and second images are fluorescent images.

In certain embodiments, the second image covers a smaller area of the biological sample compared to the area covered by the first image.

In a second aspect of the disclosure, it is provided a method of analyzing a biological sample, comprising providing an image of the biological sample as defined above, and analyzing the biological sample from the image.

In another a third aspect of the invention disclosure, it is provided a method of generating an image of a biological sample, comprising the steps of: (1) generating a first image of the said biological sample after a first protocol and before a second protocol is performed on the said biological sample; and (2) imaging a region of interest of the said sample after the second protocol has been performed on the region of interest of the said biological sample, the said region of interest being smaller than said sample.

In another a fourth aspect of the invention disclosure, it is provided a method of imaging a biological sample, comprising the steps of (1) generating a first image of the said biological sample after performing a first protocol but prior to performing a second protocol thereon; and generating a second image of a region of interest of the said sample after performing the said second protocol on the said region of interest, wherein the said region of interest is smaller than the said sample.

In another aspect embodiment of the invention disclosure, it is provided a method of

analyzing a biological sample, comprising providing a composite image of the biological sample, and analyzing the expression of the protein and the nucleic acid sequences of interest from the composite image.

In still another ~~aspect~~ embodiment of the ~~invention~~ disclosure, it is provided a kit for the fluorescent detection of a protein as well as a target nucleic acid sequence on the same biological sample. Thus, an embodiment of the ~~invention~~ disclosure provides a kit that includes components for performing the novel

method of the ~~invention~~disclosure.

In yet another ~~aspect~~embodiment of the ~~invention~~disclosure, it is provided a system for the fluorescent detection of a protein as well as a target nucleic acid sequence on the same biological sample. Thus, one embodiment of the ~~invention~~disclosure provides a system that includes means for performing the novel method of the ~~invention~~disclosure.

Brief Description of the Drawings

Figure 1 is a flowchart showing the key steps for an embodiment of the invention.

Figure 2A: Pseudocolor image illustrating a breast cancer tissue imaged at 10x. Tissue section was stained using Cy3 labeled Cytokeratin antibodies, Cy5 labeled Her2 antibody and DAPI. Immunofluorescence images were collected with monochrome camera, digitally overlaid and colored as follows: DAPI, blue; Cytokeratins, green; Her2, red. Selection of an ROI, shown in Figure 2 is shown with a light blue rectangle.

Figure 2B: DAPI and cytokeratin Images from figure 1A and an image representing background fluorescence in green channel were converted to form a virtual H&E image as described.

Figure 2C: Virtual DAB image generated from DAPI and Her2 images in 1A.

Figure 3: Combined Her2 Immunofluorescence (yellow), DAPI (blue), Her2 FISH (red) and Centromere 17 FISH (green) of a 40x ROI selected from tissue shown in Figure 2.

Detailed Description of the Invention

Definitions:

To more clearly and concisely describe and point out the subject matter of the claimed invention, the following definitions are provided for specific terms that are used in the following description and the claims appended hereto.

The singular forms “a” “an” and “the” include plural referents unless the context clearly dictates otherwise. Approximating language, as used herein throughout the specification and claims, may be applied to modify any quantitative representation that could permissibly vary