

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**

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, wherein the biological sample is a tissue sample; 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

**We claim:**

Claim 1: 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, wherein the biological sample is a tissue sample; 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.

Claim 2: The method of claim 1, wherein the region of interest is selected by comparing the first image of the biological sample to pre-determined criterion.

Claim 3: The method of any of the preceding claims, wherein the first protocol includes immunofluorescence detection of a target protein.

Claim 4: The method of claim 3, wherein the immunofluorescence detection includes physically binding an antibody with the target protein.

Claim 5: The method of claim 4, wherein said antibody is labeled with a fluorophore.

Claim 6: The method of any of claims 3-5, wherein the first protocol further includes immunofluorescence detection of at least one additional protein that provides morphological information.