Optimization of Labeling Proteins in Whole-Mount Organoids or Spheroids Using A Novel Methodology

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Introduction

Immunofluorescence labeling of organoids or spheroids is central for revealing the spatial distribution of cellular proteins (1). However, it is a laborious and time-consuming task with current technologies. Moreover, harvesting organoids and spheroids and clearing them using several reagents causes loss and damage to the sample and concludes with the deterioration of the expression of proteins(2-5). Our new technology eliminates those stressful steps and enables labeling them while still in a hydrogel (6). Furthermore, our preliminary results have shown that the delivery of primary and secondary antibodies into those 3D structures differs related to the subcellular location of the protein of interest and affects the visualization of those proteins. Therefore, in the current study, we aimed to optimize our methodology using antibodies specific to proteins located in the cell membrane, cytoplasm, or nuclei.

Methods

Liver cancer spheroids were generated and then labeled using the protocols described by Tok et al. (6). 37 primary antibodies, matching 9 secondary antibodies, 4 nuclear stains were used for immunofluorescent labeling experiments. Single- and double-protein labeling experiments were performed. Labeled samples were examined under Zeiss LSM 800.

Figure 2: Na-K ATPase in cell membrane (green) and Histone in the nuclei of dividing cells (red). Nuclear stain: Hoechst

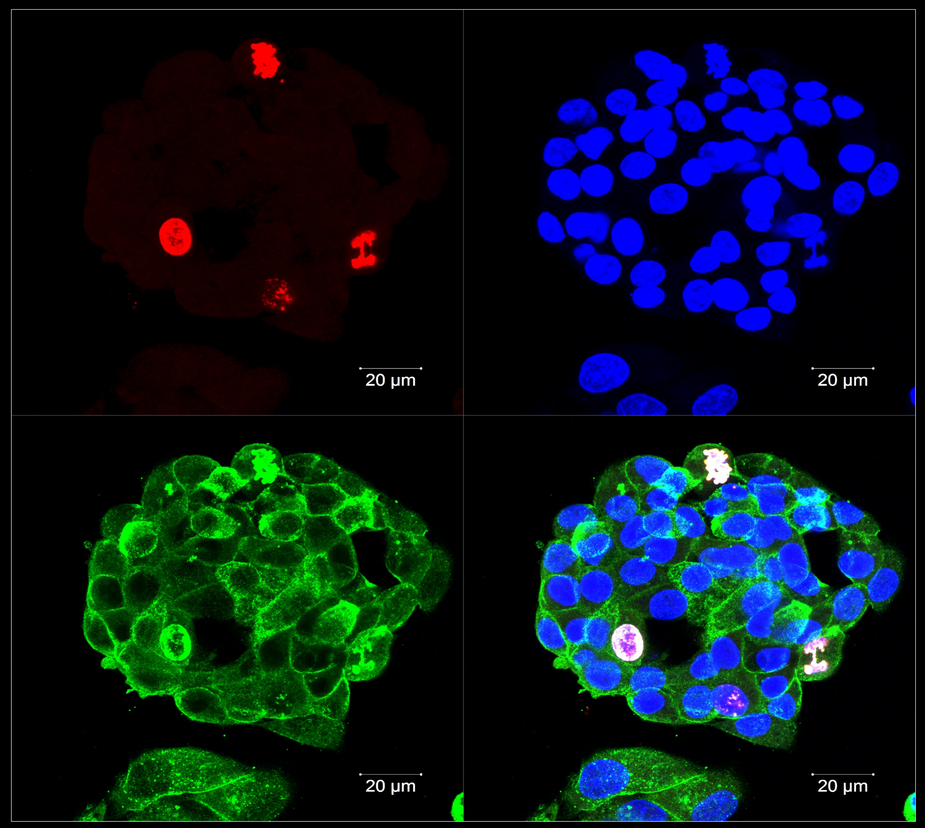
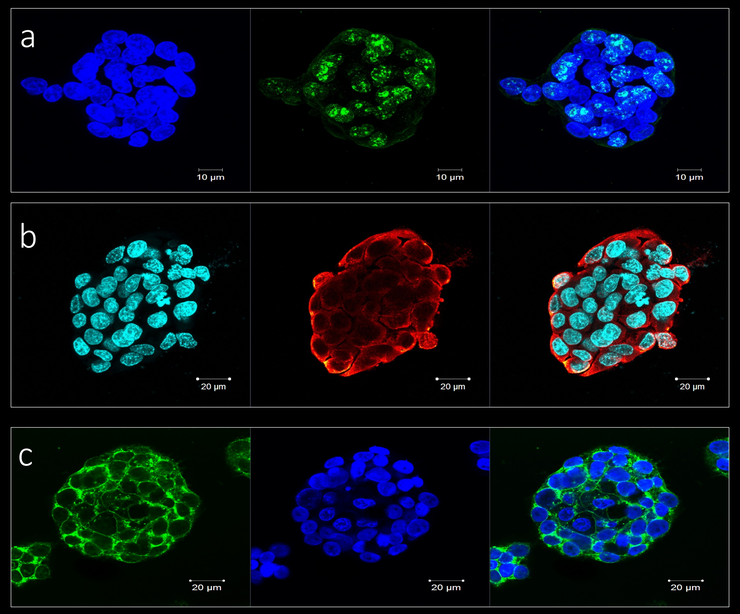


Figure 1: a. Ki67 in nuclei (green) b. E-Cadherin in cytoplasm (red) , c. Na-K ATPase in cell membrane (green) . Nuclear stain: Hoechst



Results

The most successful incubation period for primary antibodies was between 1 hrs-2.5 hrs, while we incubated the samples with the secondary antibodies between 1-1,5 hours. We could visualize proteins in the cell membrane or cytoplasm after 1,5 hours of incubation with primary antibodies and 1 hour with secondary antibodies. However, we needed to extend that period to 2 hours for nuclear antibodies. The incubation period with secondary antibodies extended to 1,5 hours when we labeled nuclear proteins (Figure 1). Similar incubation periods also enabled the visualization of two different proteins in different compartments of the cells simultaneously (Figure 2-3). We needed to adjust the incubation period for spheroids that were around 1mm. We could see the antibodies' penetration into the spheroids' center in those larger spheroids after increasing that duration to 30 min- 1 hr for primary and 30 min for secondary antibodies. The total time for immunolabeling experiments was eight hours or less in different experimental designs.

References

1. Rios AC et al.. Imaging organoids: a bright future ahead. *Nature Methods* **15**, 24–26 (2018).
2. Dekkers JF et al. High-resolution 3D imaging of fixed and cleared Organoids. *Nature Protocols* **14**, 1756–1771, (2019).
3. Renner H et al. Fluorescence-based Single-cell Analysis of Whole-mount-stained and Cleared Microtissues and Organoids for High Throughput Screening. *Bio-protocol* **11(12)**, 4050 (2021).
4. Edwards SJ et al. High-Resolution Imaging of Tumor Spheroids and Organoids Enabled by Expansion *Microscopy* **7**, 1-10 (2020).
5. Bergdorf KN et al. Immunofluorescent staining of cancer spheroids and fine-needle aspiration-derived organoids. *STAR Protocols* **2(2)**, 100578 (2021).
6. Tok OE et al. Culturing, Culturing, Freezing, Processing, and Imaging of Entire Organoids and Spheroids While Still in a Hydrogel. J. Vis. Exp. (190), e64563, doi:10.3791/64563. 2022.

Conclusions

Experiments with 37 primary antibodies demonstrate that new technology enables the visualization of several proteins simultaneously in intact 3D structures while eliminating antigen retrieval, harvesting, and clearing steps. The sample is still in its surrounding environment, i.e., hydrogel, at the end of the experiments, and that does not cause any additional background noise in that method. Nuclear proteins can be labeled after more extended incubation periods. Since organoids and spheroids can be very dense, with several cell layers, extended incubation periods are also required with reagents to penetrate their core for large specimens. With the new method, antibodies successfully penetrate the center of the structure. The incubation period must be adjusted according to the protein's location in the cell. The reliability and reproducibility of the results increase since the 3D structure is protected in its growing environment during the entire experiment. The omission of the stressful steps and the elimination of pipetting, transferring, and centrifuging speed up the labeling process and reduce human errors. While current wholemount immunofluorescence labeling protocols take three days to weeks (1-5), the total immunolabeling procedure with that new method does not take more than 8 hours.

Figure 3**:** Na-K ATPase in cell membrane (green) and Albumin in cytoplasm(pink). Nuclear stain: DAPI