Best staining practices

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1. Introduction

A live cell is transparent and exhibits no contrast for classical microscopy. However, cells feature a valuable natural physical marker: the refractive index (RI). The RI describes how the medium impacts the light propagation. Having a different composition, the inner parts of the cell have slightly different refractive indexes.

The 3D Cell Explorer can detect this differences and delivers a 3D reconstruction of a given volume by measuring the RI point by point. Based on this information organelles or compartment are segmented thanks to a process called digital stain.

This document is a practical guideline about how to digitally stain your sample obtaining the best possible results. It contains i. a detailed description of the procedure to follow to digitally stain with our software STEVE; ii. a short theoretical explanation about the parameters (Refractive Index and Index Gradient) on which the digital staining is based, and iii. some examples of stain manipulation. Finally, the document presents iv. a few "gold" advices to improve your staining and avoid typical mistakes.

All the data used in this document are available on http://nanolive.ch/downloads/data/ or from the "Help > Get more data" menu in STEVE.

## 2. How to digitally stain (d-stain) cells with STEVE

2.a STEVE legend (Figure 1a)

2.   How to digitally stain (d-stain) cells with STEVE

2.b   Key Steps

1.   Choose a meaningful slice on the RI-map: Z-stack panel. (Figure 1b)
    a.   Move through the cell in z by dragging the mouse up & down while keeping the left button pushed or just use the "Slices slider"

2.   Pick a new stain. (Figure 1c)
    a.   Click on the + button

3.   Choose a name (optional). (Figure 1d)
    a.   Click on the black-gray line edit form.
    b.   Write down the name in the line text input.

4.   Choose a color. (Figure 1e)
    a.   Click on your desired color.
    b.   Option: drag the mouse to have a more precise vision of the color that you could select.

Figure 1b   Figure 1c   Figure 1d   Figure 1e
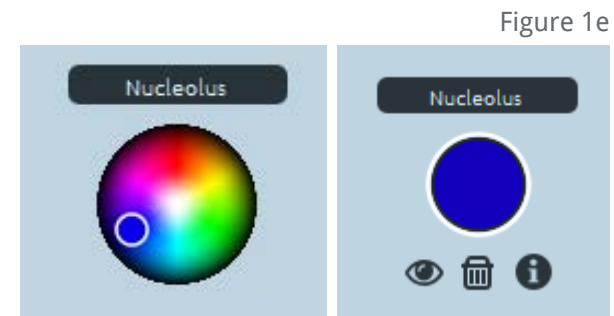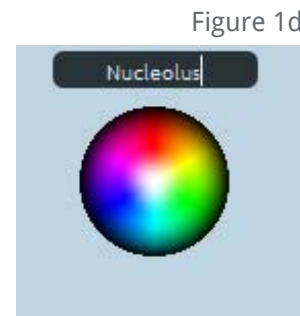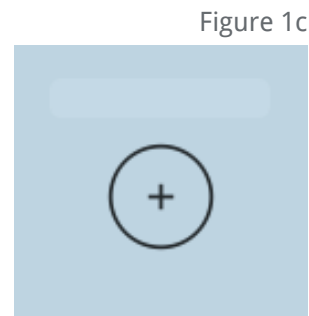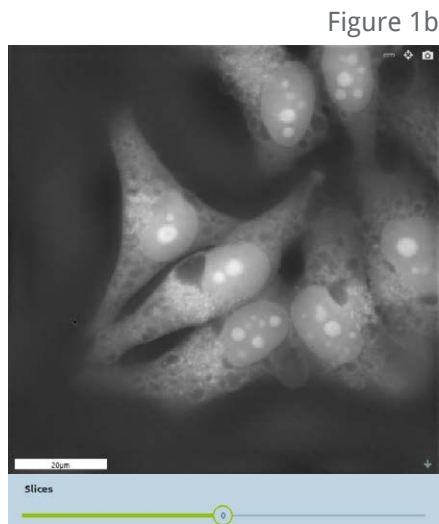
5.   Go to the RI-map: Z-stack panel and draw. (Figure 1f)
   a.   Click and/or drag the mouse on your desired region of interest. The pixels under your cursor are glowing in the panel view. When you release the mouse, we compute the stain from your selected pixels.
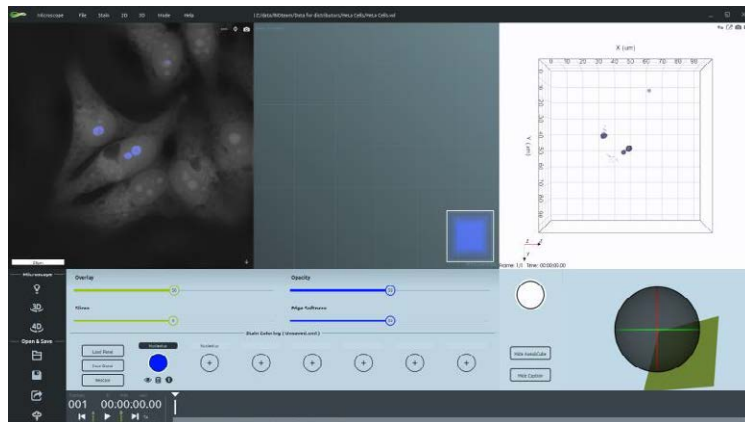   b.   Option: You could zoom in on the RI-map: Z-stack panel with the wheel of the mouse.

Figure 1f
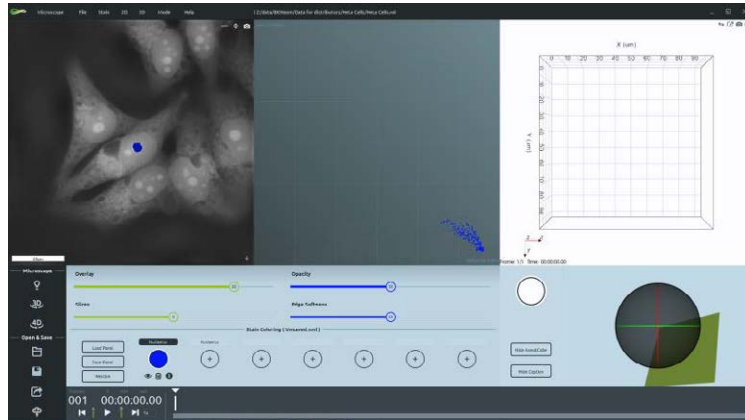
6.   Now the stain is represented in the D-stain panel view and superimposed on the RI-map: Z-stack panel. (Figure 1g)
   a.   Option: you could change the weight of the colored image on the RI-map: Z-stack panel by moving the Overlay slider.
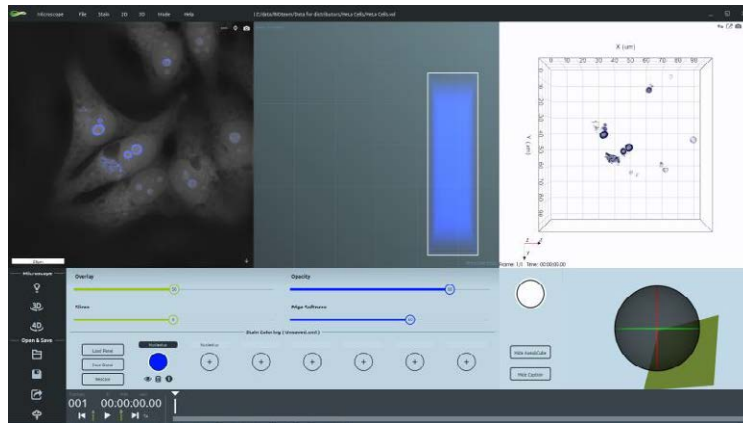
Figure 1g

7.   Manipulate your stain (Figure 1h):
   a.   Change the opacity (Opacity slider or drag up-down with the right click pushed).
   b.   Change the edge softness (Edge softness slider or drag up-down holding the right click inside the stain edge).
   c.   Move a stain in the panel view (click on the stain and drag it on the desired position in the RI –Index gradient space).
   d.   Change the shape of the stain (select an edge or a corner and drag it).

Figure 1h

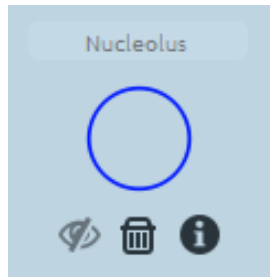**8.   To add other stains repeat the above steps. (Figure 1i)**

Figure 1i

9. **Stain's options:**

   a.   Enable/Disable the visualization of the stain (Figure 1j)
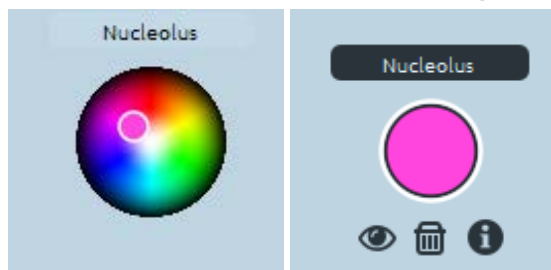
Figure 1j

   b.   Delete the stain (Figure 1k)

Figure 1k

   c.   Change the color (by double clicking the colored button or by clicking on the palette icon) (Figure 1l)

Figure 1l

3. What is the "Refractive Index" (RI) [horizontal-axis] and the Index Gradient [vertical-axis]?

The refractive index (RI) of a medium is defined as the ratio between the speed of light in vacuum and the speed of light in that medium.

The formula is: $\quad \mathbf{RI} = \dfrac{\mathbf{c}}{\mathbf{v}}$

where the speed of light is approximatively **c** = 300,000 km/s and **v** is the speed of light in the desired medium.

The Refractive Index for water is therefore: $\quad \mathbf{RI_{water}} = \dfrac{\mathbf{300.000\ km/s}}{\mathbf{225.000\ km/s}} = \mathbf{1,\overline{3}}$

Some typical RI values evaluated using green light (520 nm) are reported in **Table 1**.

The **Index Gradient** (**|∇RI|**) describes how the Refractive Index varies in the proximity of a pixel.

The formula is: $\quad \left|\nabla RI\right| = \sqrt{\left(\dfrac{RI_{x+1,y,z} - RI_{x-1,y,z}}{2p_x}\right)^2 + \left(\dfrac{RI_{x,y+1,z} - RI_{x,y-1,z}}{2p_y}\right)^2 + \left(\dfrac{RI_{x,y,z+1} - RI_{x,y,z-1}}{2p_z}\right)^2}$

where $p_{x,y,z}$ is the resolution, and $RI_{x\pm1,y\pm1,z\pm1}$ is the RI value of the neighbouring pixels (**Figure 2a**).

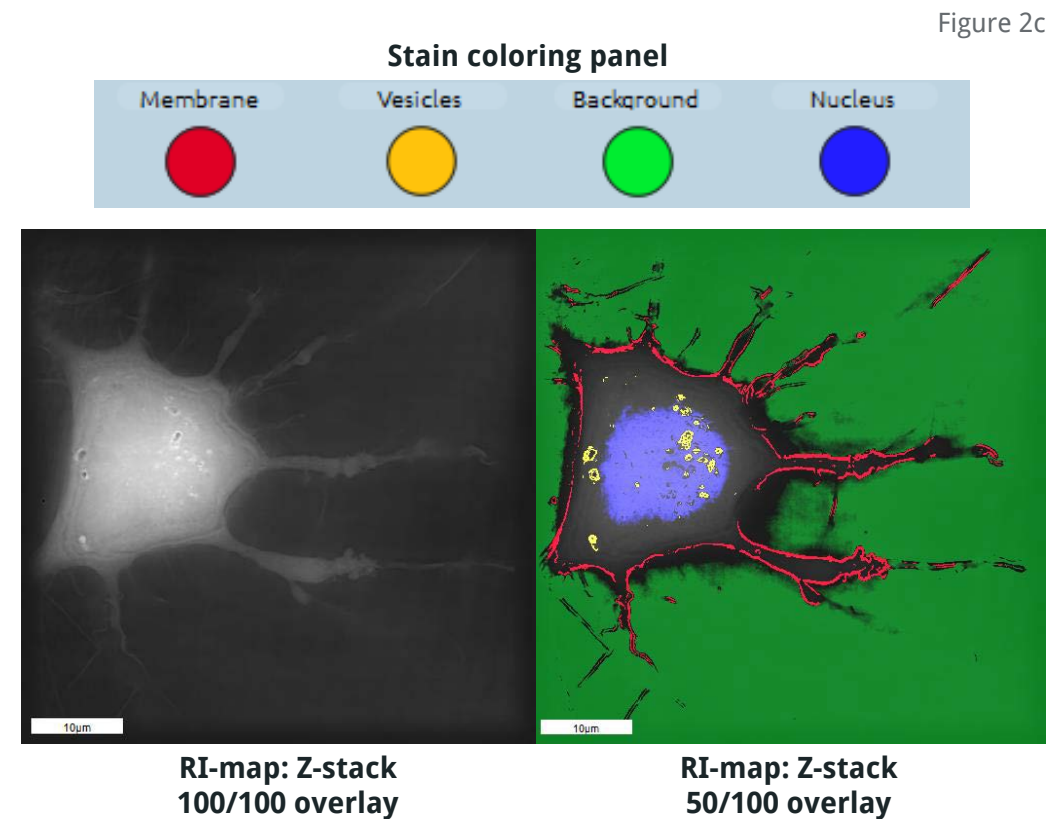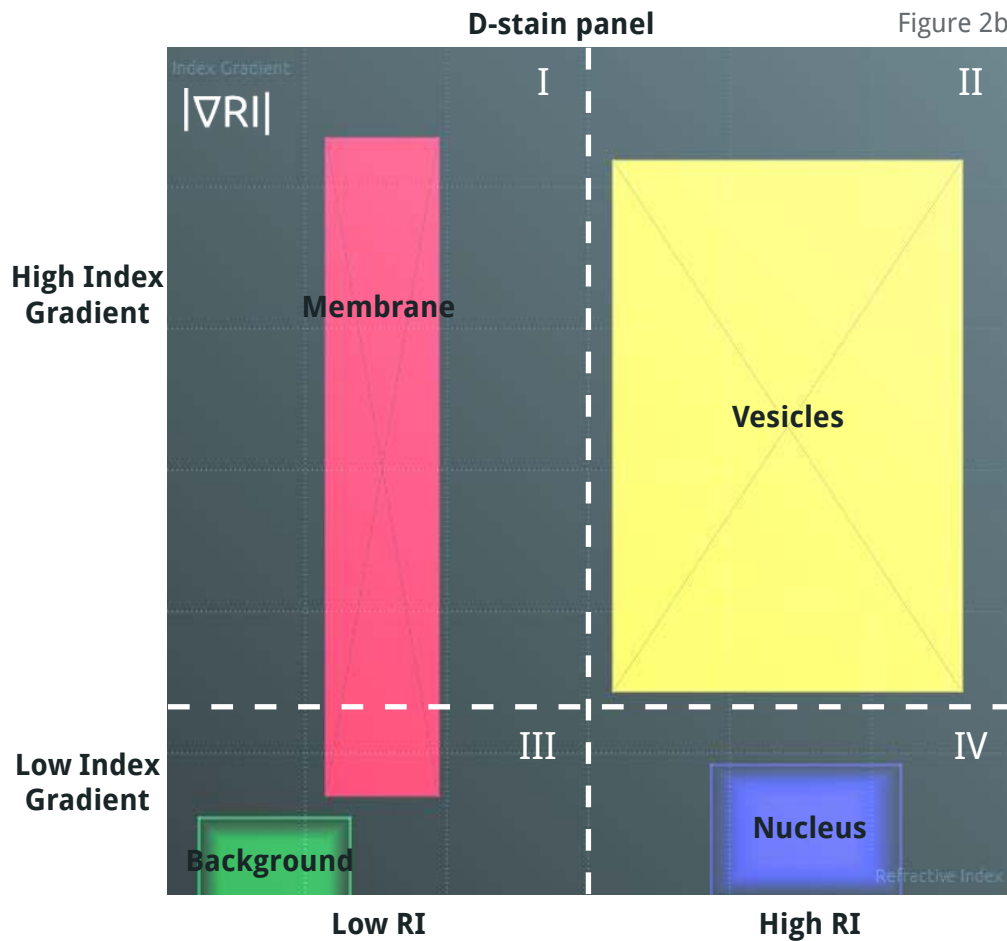| Medium	RI
Water	1,333
PBS	1,334
DNEM + 10% FBS	1,337
Ethanol	1,364
Urea	1,495
Fluorodish glass	1,525

Table 1: RI value of typical media under green light (520 nm)
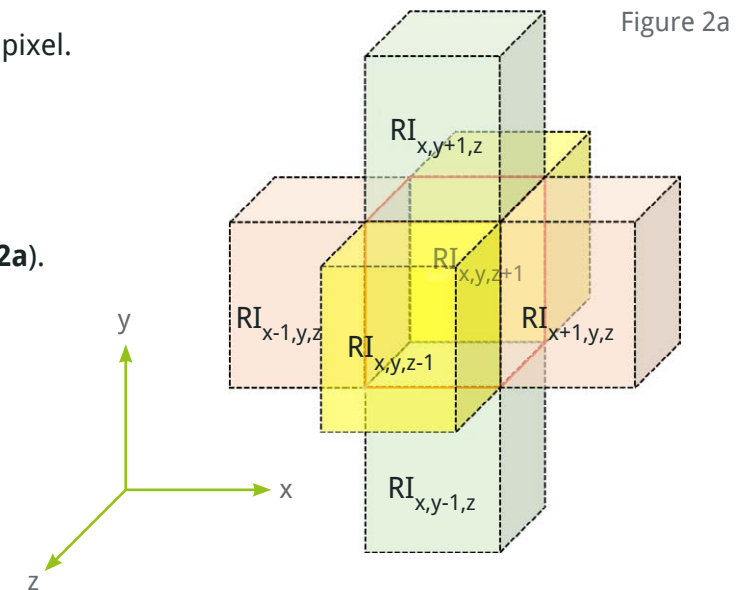
Figure 2a

The D-stain panel in **Figure 2b** shows the typical disposition of the stain squares:

- **Quadrant I** is characterized by low RI but high RI gradient, this region is typical for edge structures, like membranes.
- **Quadrant II** has high RI values and high RI gradient, it generally includes texture zones, like vesicles.
- **Quadrant III**, having a low RI and low RI gradient, is more transparent and homogeneous and is typical for the background.
- **Quadrant IV** is characterized by high RI and low RI gradient, generally includes high density homogeneous structures like the nucleus.

The D-stain applied on the RI-map of cell data is shown in **Figure 2c**.

D-stain panel — Figure 2b

Stain coloring panel — Figure 2c

RI-map: Z-stack 100/100 overlay

RI-map: Z-stack 50/100 overlay

4. Stain Manipulation

4.a   Position

i.   **Shift the staining square horizontally** to modify the RI range and stain a different part of the sample (**Figure 3b**). Different biological compartments can be distinguished by their specific RI range (cellular membrane has different RI range compared to nuclear membrane).

ii.   **Shift the staining square vertically** to modify the Index Gradient range (**Figure 3c**).

Figure 3d shows the combined effect of these position manipulations.
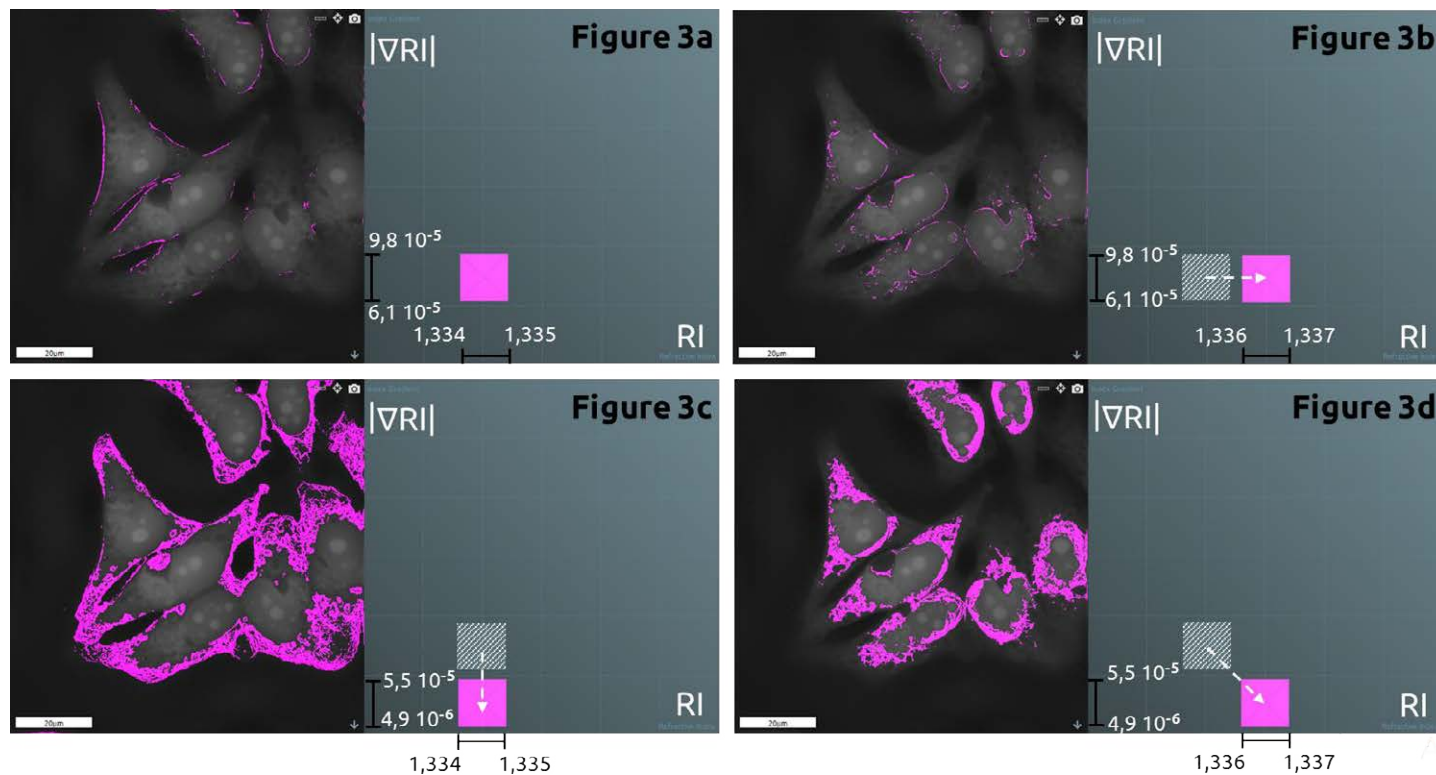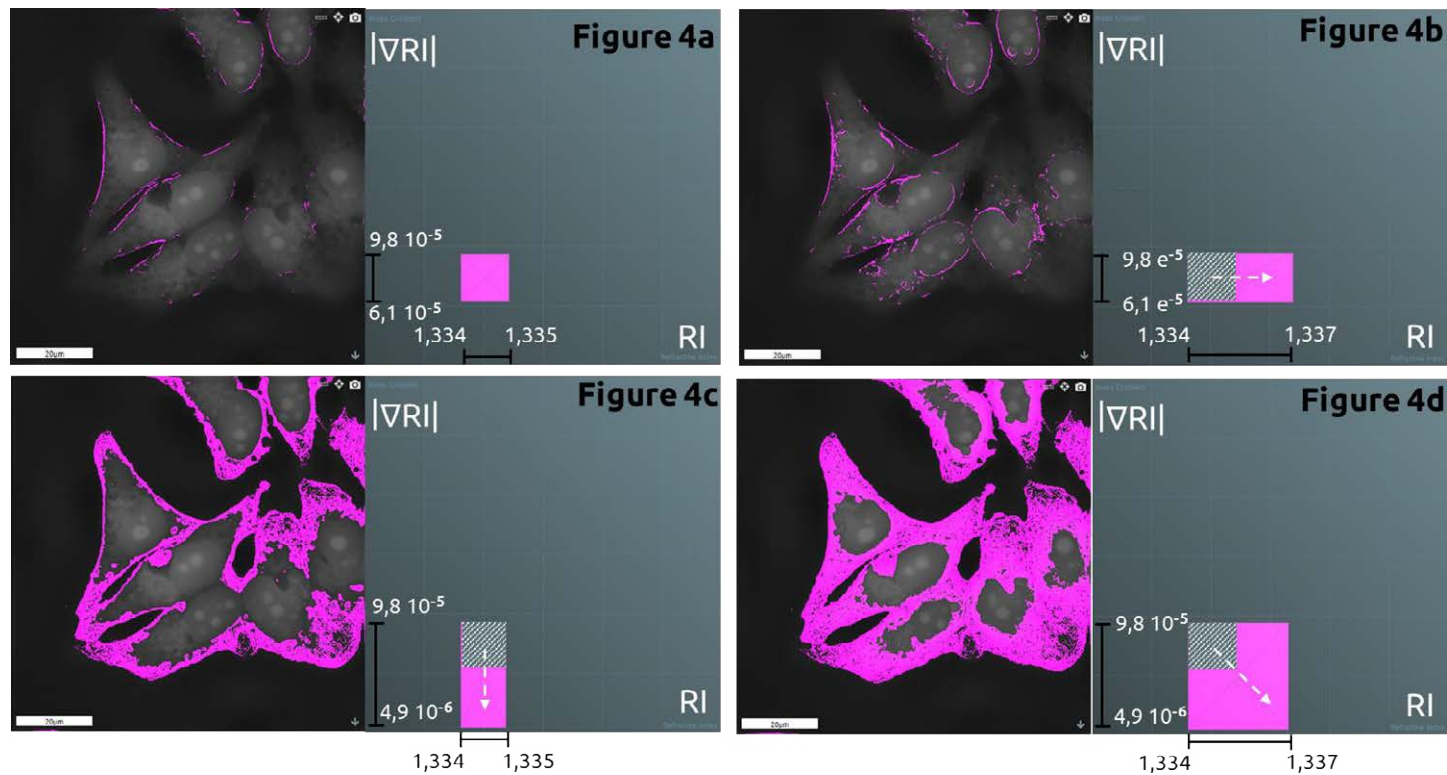
4. Stain Manipulation

4.b Shape

i. **Enlarge or reduce the width of a staining square** to stain respectively more or less sample structures (**Figure 4b**).

ii. **Enlarge or reduce the height of a staining square** to integrate more or less variations (**Figure 4c**)

**Figure 4d** shows the combined effect of these shape manipulations.
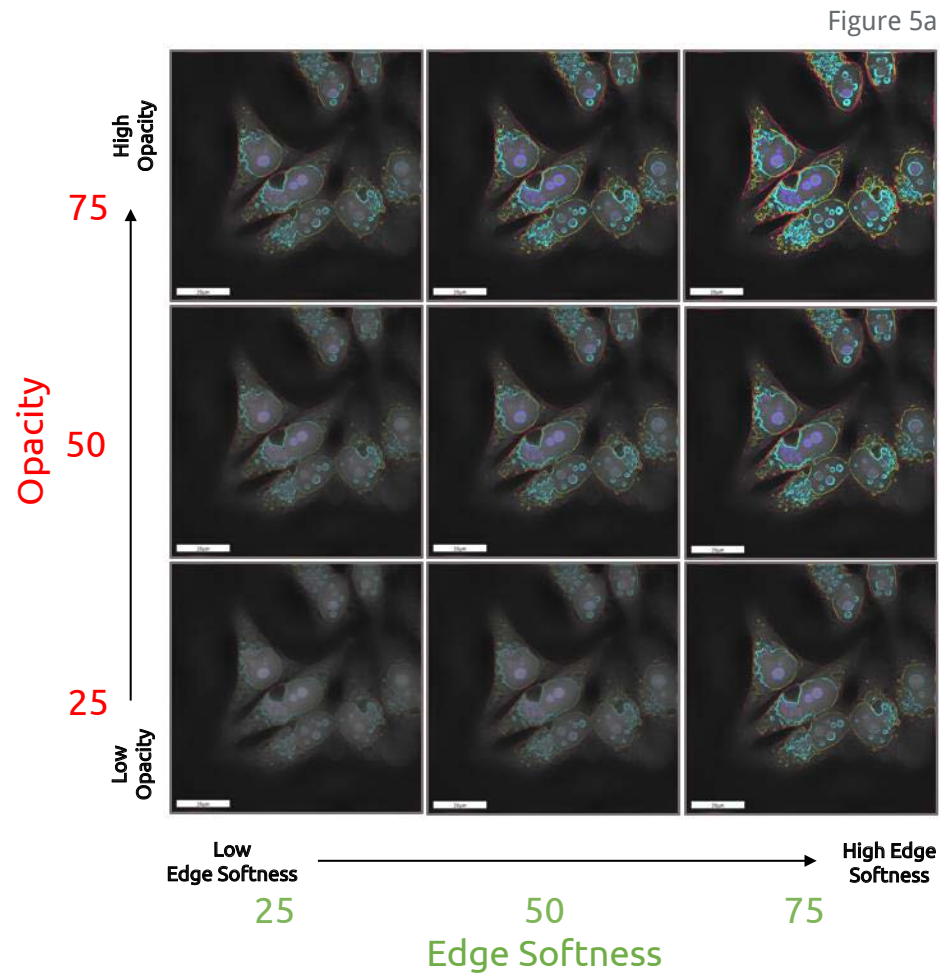
4.   Stain Manipulation

4.c   Opacity & Edge softness

i.   **What does opacity mean?**
        The opacity parameter sets the maximum intensity of the colored pixels.
        Minimum level is 0 (transparent), maximum level is 100 (opaque).

ii.   **What does edge softness mean?**
        The edge softness parameter sets how fast the intensity changes inside the colored pixels.
        Minimum level is 0 (smooth change), maximum level is 100 (quick change).

iii. **How do these parameters affect your image?**
The panel shows the effects of Opacity & Edge softness manipulation on the RI-map: Z-stack (**Figure 5a**) and d-stain panel (**Figure 5b**).
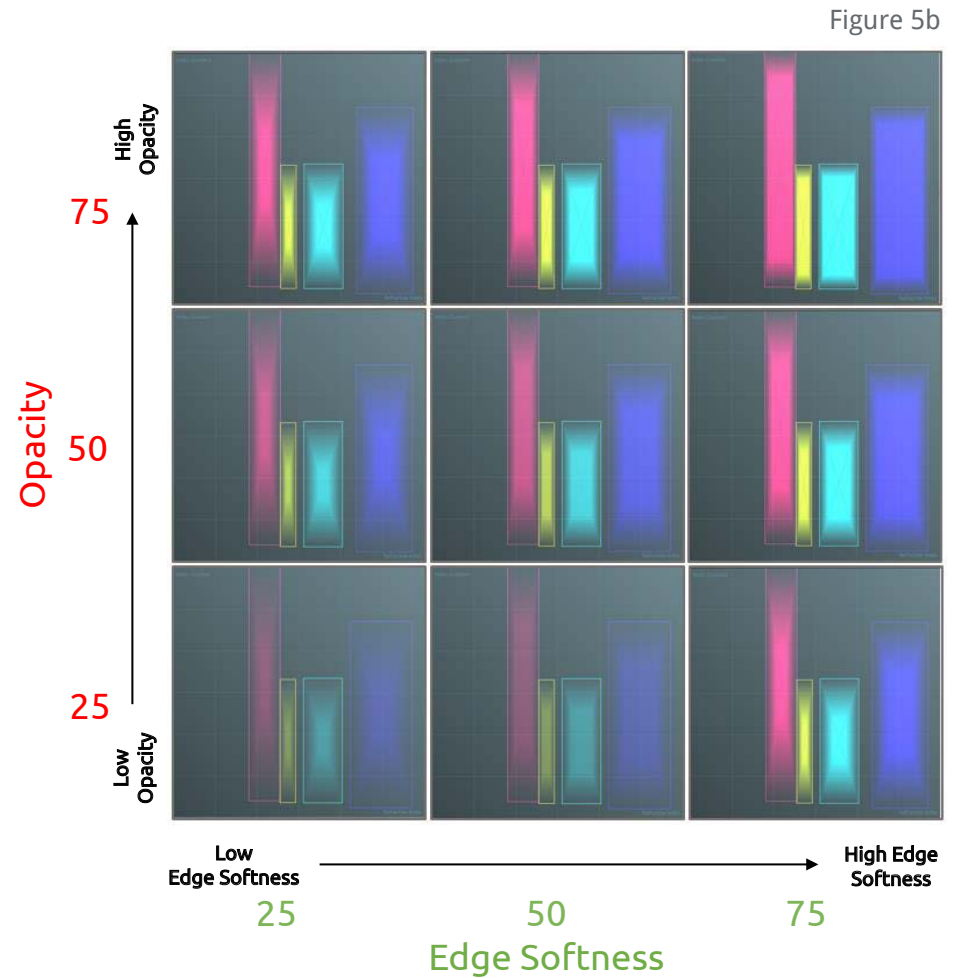
Figure 5a

Figure 5b

The panel shows the effects of Opacity & Edge softness manipulation on the 3D Visualization - top view (**Figure 5c**) and 3D Visualization - lateral view (**Figure 5d**).
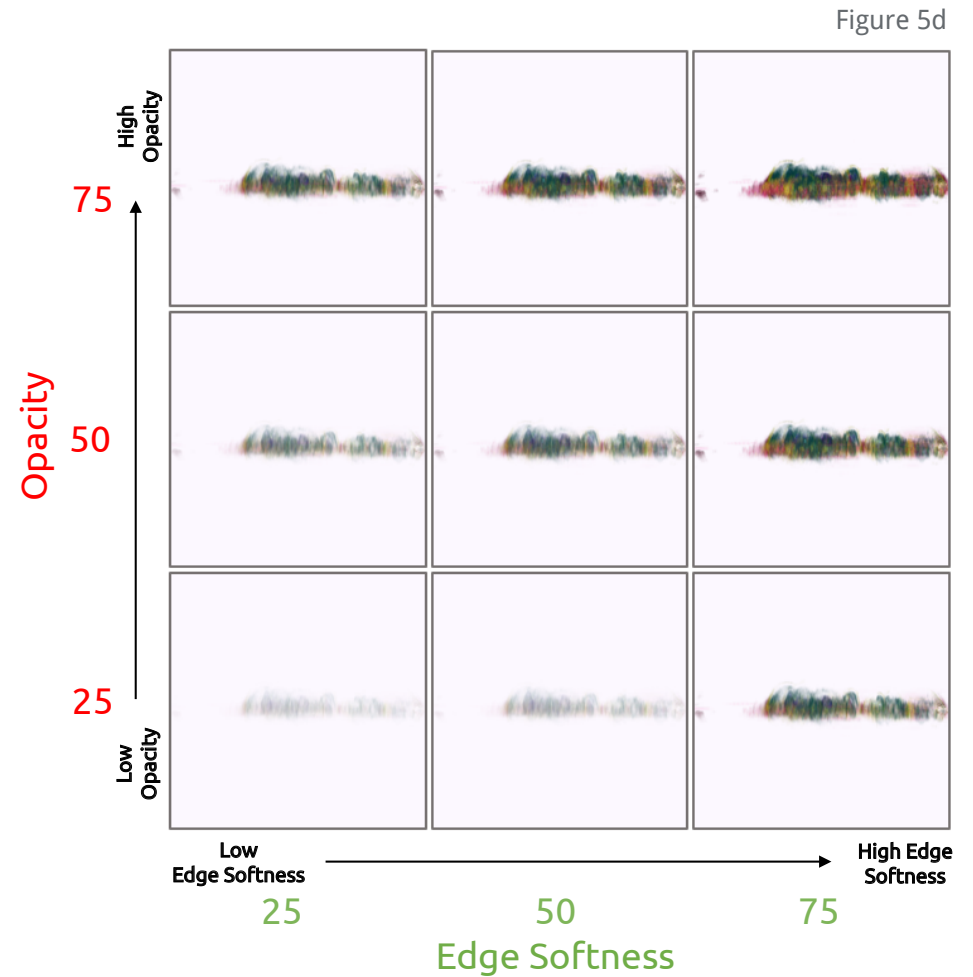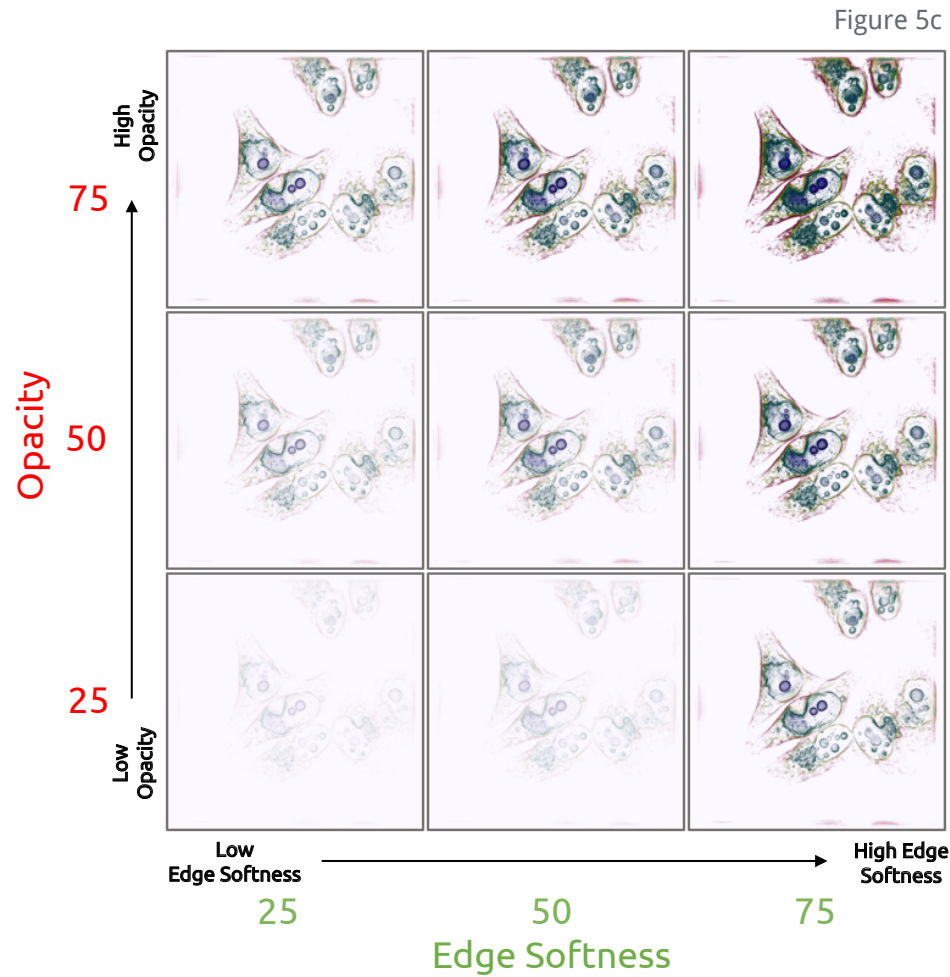
Figure 5c

Figure 5d

5.   Best staining practices

5.a   Avoid imaging artifacts

If a stain square overlaps with the background region, some imaging artifacts will appear on the 3D Visualization. Imaging artifacts are pointed by circles in **Figure 6a**: the stain covers the background too and it is spread up and down the plane where the cells are adhering (green line).
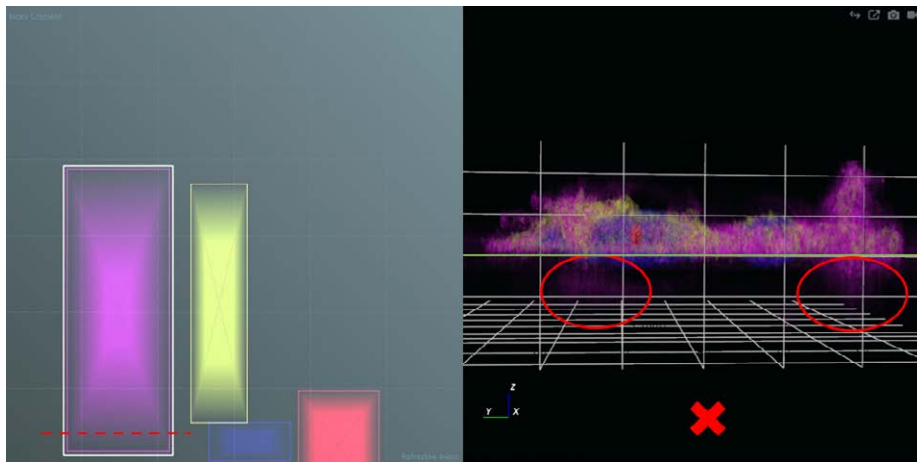

Figure 6a

To only digitally stain meaningful sample regions, make sure that all your stain squares do not overlap the background region (**Figure 6b**).
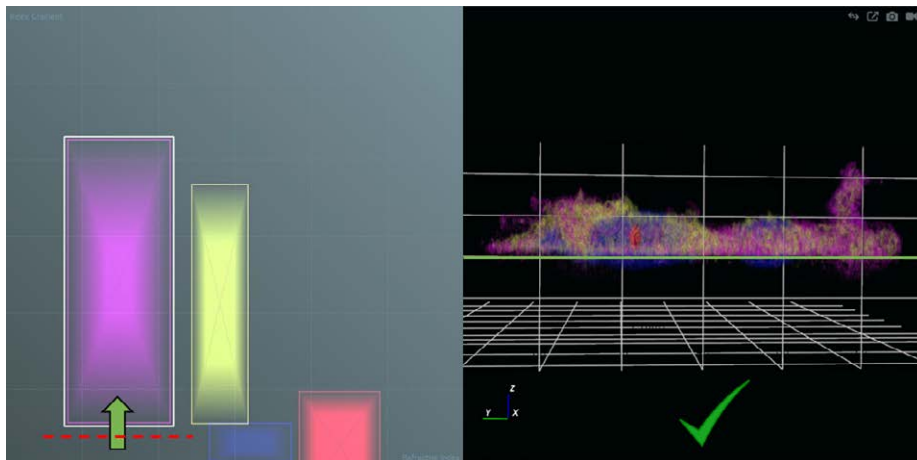

Figure 6b

5.   Best staining practices

5.b   Avoid monochromatic staining

If you want to efficiently distinguish different sample structures, avoid the use of identic or similar colors (**Figure 7**).
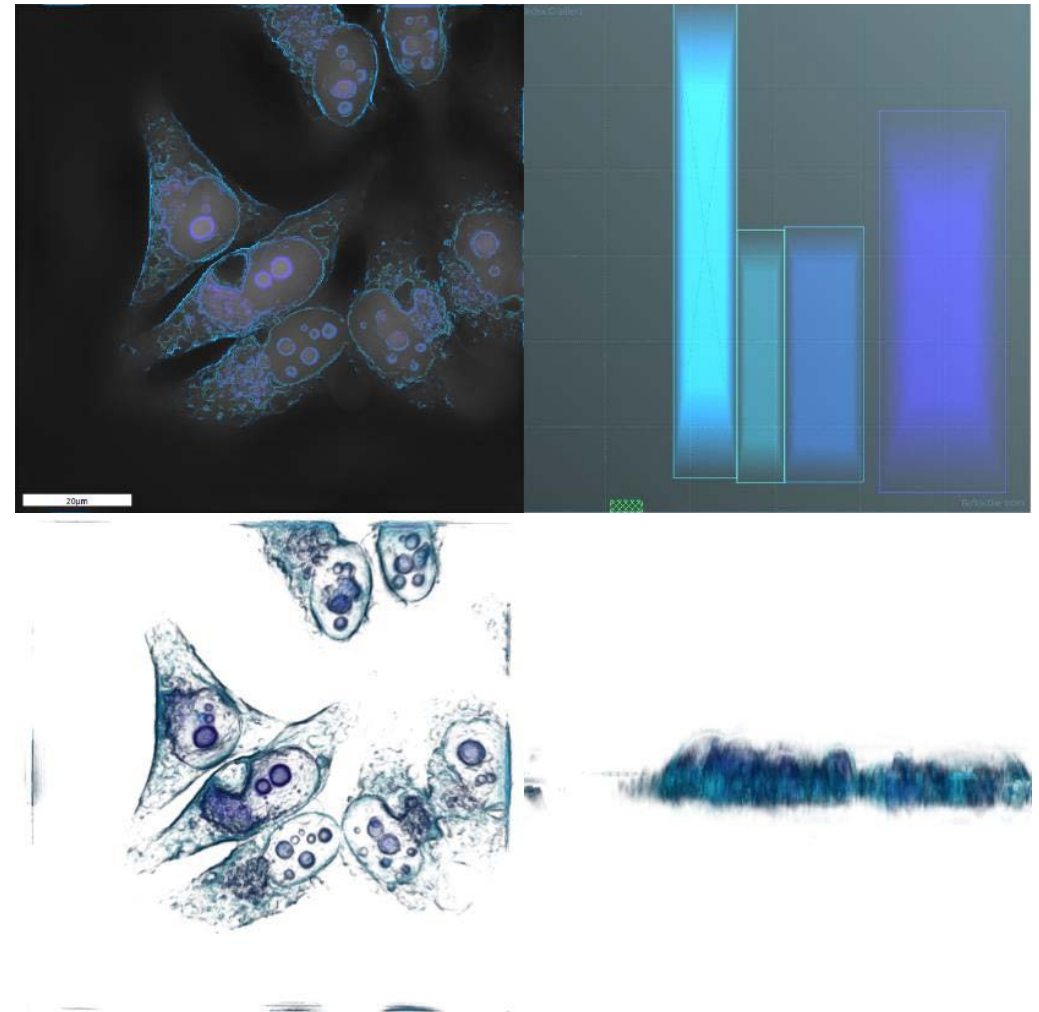
Figure 7

5.   Best staining practices

5.c   Overlapping stain squares

Generally a simple and efficient digital staining panel includes a few and well distinguished stain squares (not overlapping) like the reference staining (to be loaded in STEVE directly).

i.   Anyway, a sub-stain (**Figure 8a**) could be used to put in evidence structures inside of a bigger compartment characterized by a wide RI range e.g. stain organelles and the whole cell at the same time.
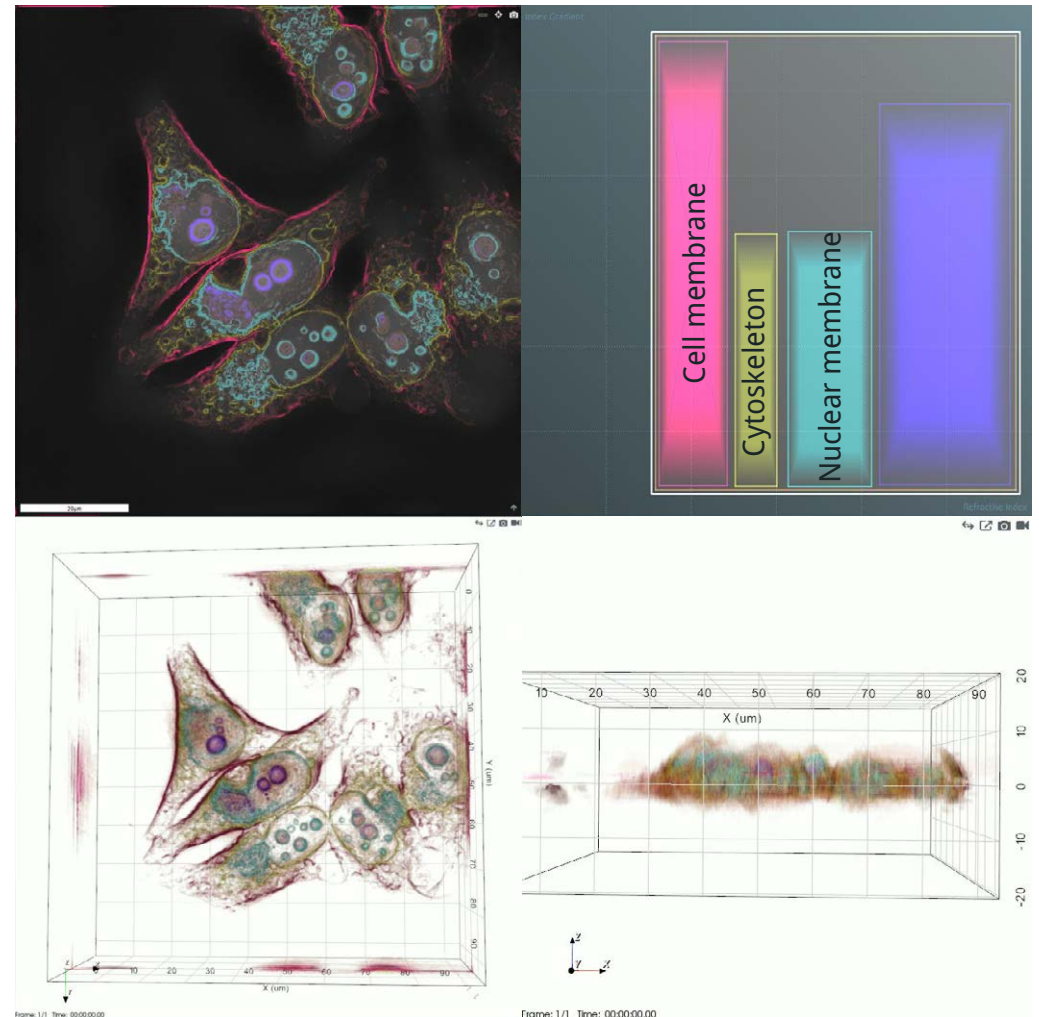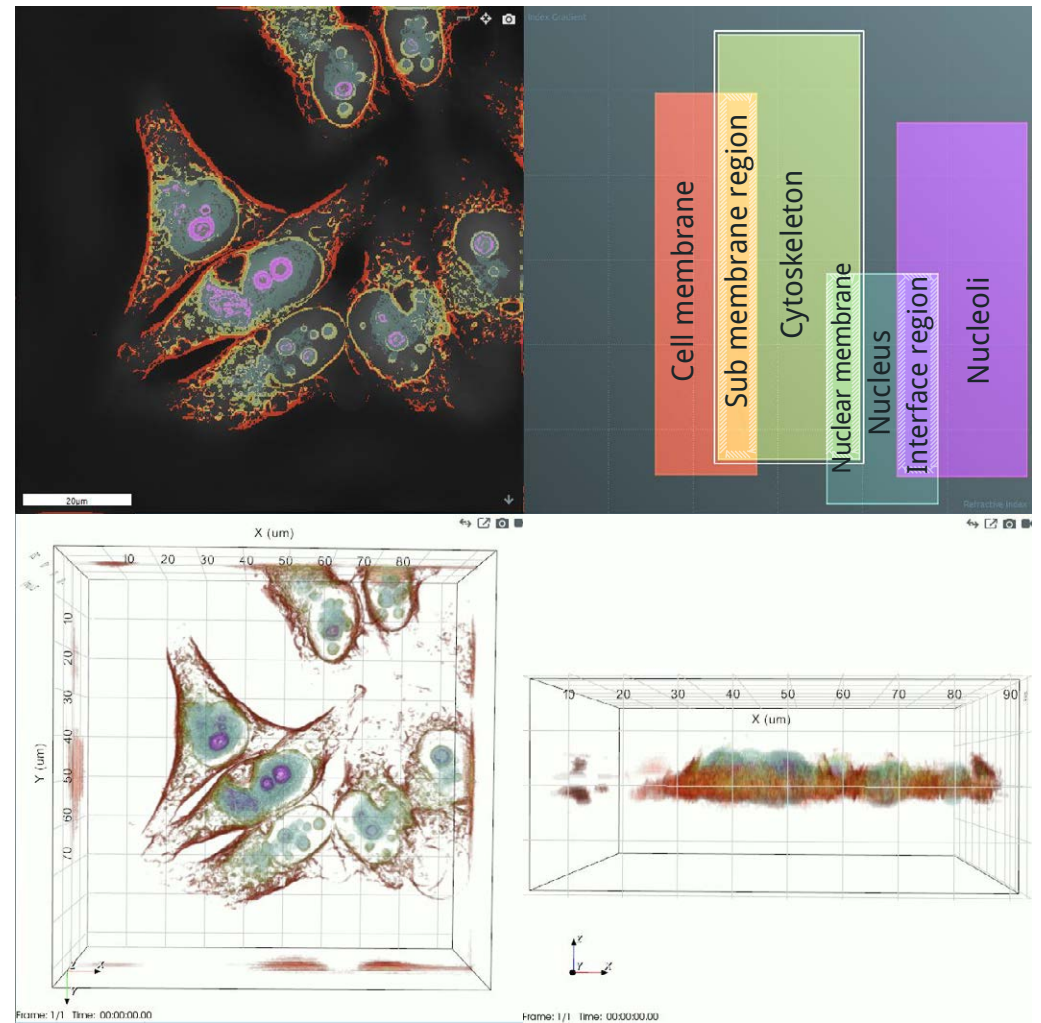
ii.   In particular cases, several overlaying stains (**Figure 8b**) allow to put in evidence regions that would otherwise be difficult to stain.

5. Best staining practices

5.d How to stain all the pixels with a specific RI value (e.g. membranes)

Create a stain square as narrow as possible around the target RI value (smallest RI range), as high as the total panel (no discrimination based on the Index Gradient) (**Figure 9**). Moreover, to put in evidence all the stained pixels, it is suggested to set the "Opacity" and "Edge softness" parameters at their maximal levels (100).

Figure 9
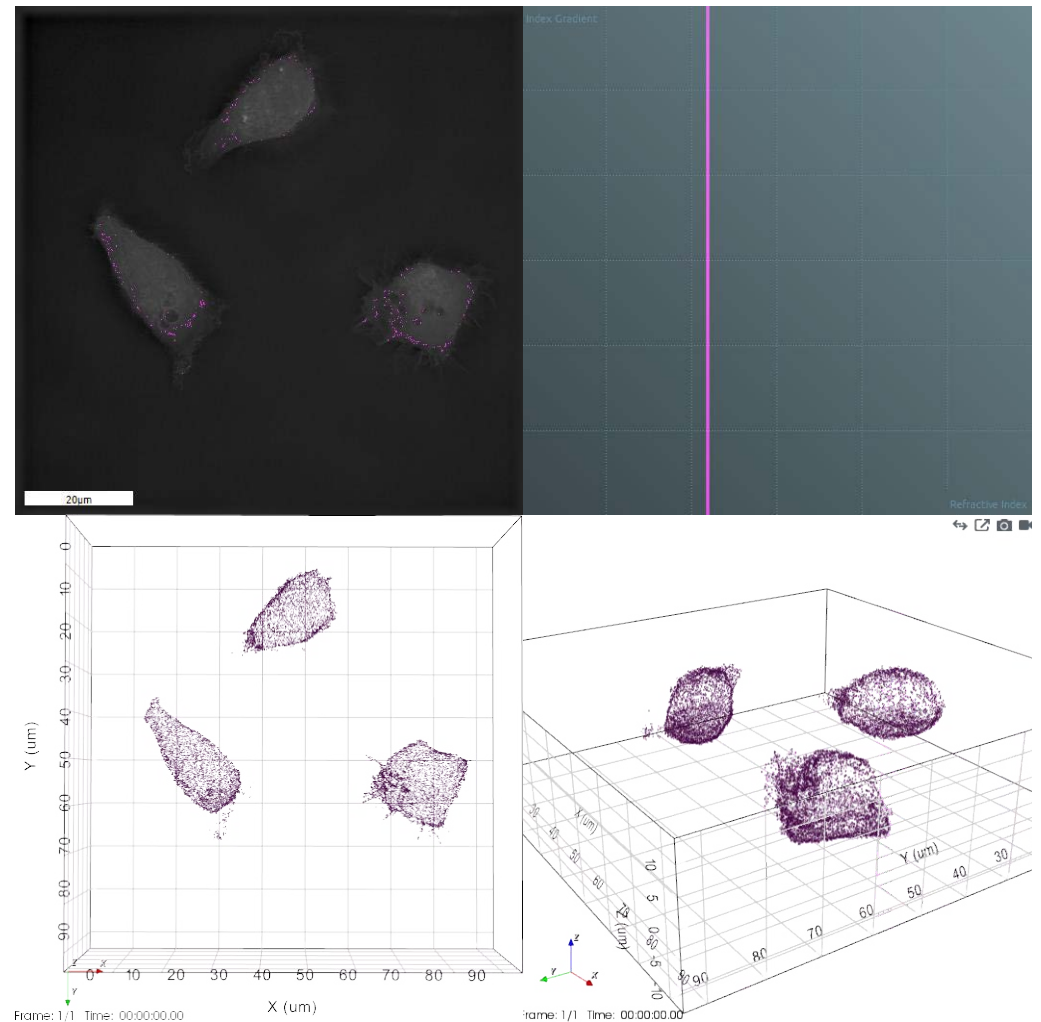
6. Conclusion

The digital staining is an easy and understandable procedure but as many other lab practices require time and experience to be improved to the maximum level. We believe that this document will be able to help you to take a terrific shortcut on this learning path. For any further clarification please check our supporting material or contact our support team at **support@nanolive.ch**.