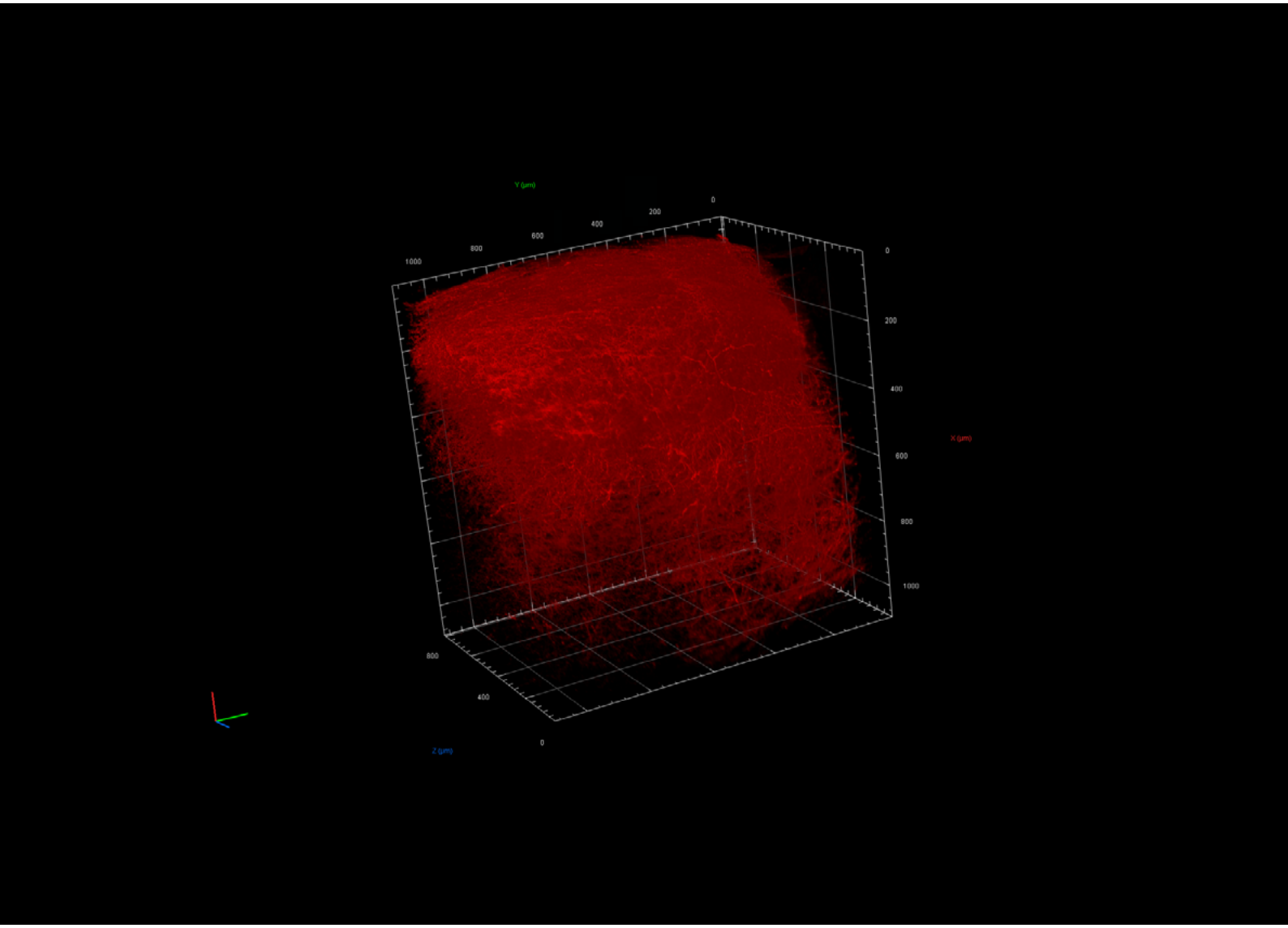
Application Note



Adjusting Refractive Index for Clearing Applications

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Adjusting Refractive Index for Clearing Applications

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Task

When scientists starts working with clearing applications and adapting the clearing procedures to their specimens, there are many things to consider, like specimen integrity, fluorescent labelling and also refractive indices (R.I.). The main goal of the clearing procedure is to equilibrate the refractive index of the specimen to the imaging medium. Since the sample will be imaged with a microscope, the refractive index of the medium should match as closely as possible the used optics as well. Therefor it is crucial to check the refractive index of clearing solutions to minimize mismatches and resulting optical errors, such as spherical aberrations, and to increase the image quality and depth penetration, before and during the clearing procedure.
For imaging of cleared specimen with Lightsheet Z.1 the refractive index of the imaging medium also influences the beam waist position of the light sheet, which should therefor match to the optics and used sample chamber to ensure that the minimum beam waist is positioned close to the optical axis of the detection optic.
In this technology note we describe a simple method how to check the refractive index for clearing and imaging solution necessary for successful clearing imaging with Lightsheet Z.1.

Methods and material

Refractometer to measure refractive indices of clearing solutions: e.g. Krüss HR 901. Universal hand refractometer with stage switch for all ranges. Adjustable prisms for sharp contours, direct and indirect light guidance for measurement of clear and opaque substances. With thermometer.

http://www.kruess.com/laboratory/products/refractometers/manual-handheld-refractometers/

Specifications:

- Measurement range: 1.333 – 1.517 nD
- Accuracy: 0.0005 nD

Figure 1 *Krüss HR 901 hand refractometer*

To avoid surprises at the microscope it is helpful to measure the refractive index of all clearing solutions to be used. This way one can monitor that the created sample will match the optical characteristics of the imaging system. The easiest and cheapest way to do so, is using a hand refractometer, where you have to place a drop of the solution on the corresponding prism. The refractive index can be read of the instruments scale, at the position of solution/prism interface.
There are also automatic refractometers available, into which a drop of the solution is placed and the R.I. will be displayed immediately.

Ideally the temperatures of the used clearing solutions should remain stable between setting the R.I. and imaging as the refractive index can be influenced by temperature changes. Equilibrating a sample to a certain refractive index can take several days or even weeks depending on the sample size and density. Therefor it is crucial to start the

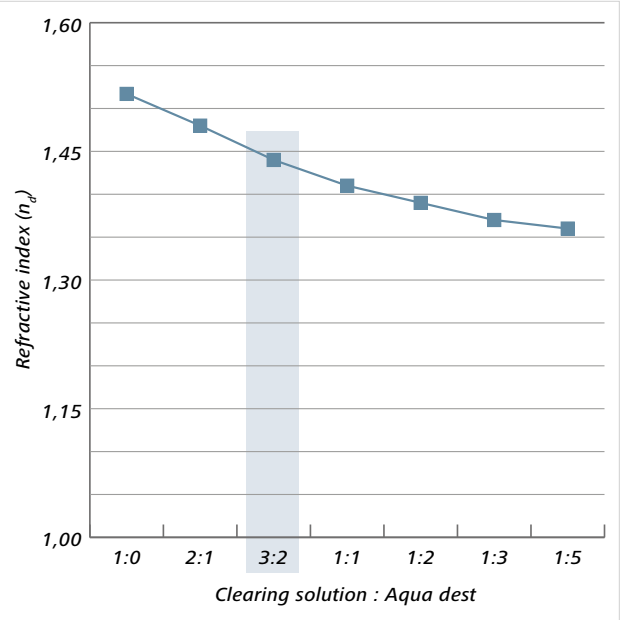

Figure 2 *Minimizing refractive index of a clearing solution with a dilution row using increasing amount of distilled water.*

clearing procedure with controlled solutions and measure the R.I. from time to time during the clearing procedure. For all water based clearing methods like Scale[1], CLARITY[2] and CUBIC[3] the easiest way to lower the refractive index from a value that is higher than desired, is a dilution of clearing solution with distilled water. Of course the same procedure can be done with other solutions, depending on the clearing protocol.

In the experiment described here, we changed the refractive index of Clarity imaging medium from > n_d =1.512 to < n_d =1.49 using distilled water to match the Lightsheet Z.1 optics. To do so, a serial dilution of the clearing medium with distilled water was set up. After each dilution step the refractive index was measured using the above mentioned refractometer. The 20x Lightsheet Z.1 detection optics for imaging samples with n_d= 1.45, such as CLARITY (Clr Plan

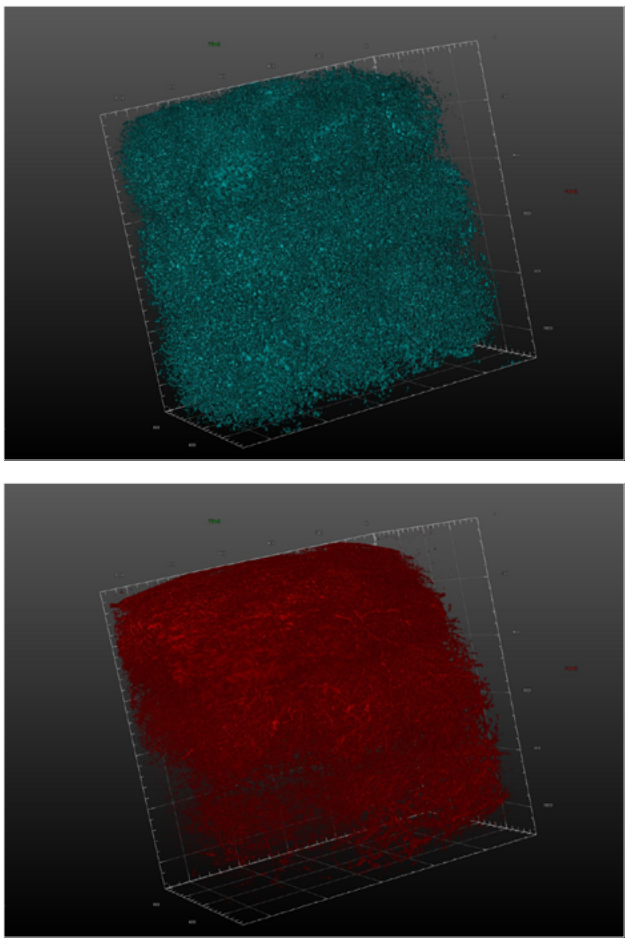

Figure 3 *Mouse brain cleared with CLARITY. Sample stained with Tyrosine hydroxylase (Cy3) and counterstained with Hoechst. 3x3 Tiles acquisition with 405 nm and 561 nm excitation respectively, single side illumination. Lightsheet offset position was adjusted for tiles positions far away from sample surface.*
Image courtesy: Prof Sun Woong, Korea University, Department of Anatomy

Neofluoar 20x/1.0 Corr n_d=1.45), is equipped with a correction collar for adjusting refractive index mismatches of ± 0.03. So there is no need to match the refractive indices perfectly to n_d= 1.45 respectively.

Results

By using a dilution ratio of 3:2 (Clearing medium : aqua dest) we shifted the initial refractive index from n_d= 1.512 to n_d=1.446, to match the refractive index working range of the optics (Fig. 2).

Fig. 3 and 4 shows renderings created with Arivis Vision 4D from a mouse brain sample cleared with CLARITY. In the described case, the imaging conditions were not optimal as the refractive index equilibration was done after the clearing procedure was completed. But after keeping the samples in this new medium for over an hour to equilibrate, imaging depth deeper than 1.7 mm could be reached. If the incubation time is not long enough, refractive mismatches in the sample might influence imaging quality and will lead to poor results. That´s the reason why setting the right refractive index of the solution should be done ideally before the clearing procedure is started.

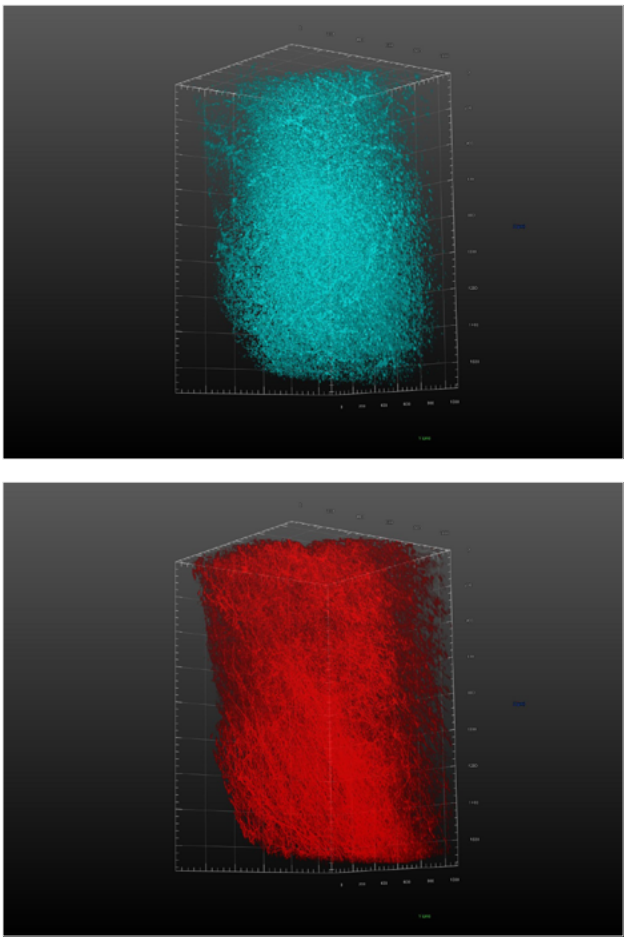

Figure 4 *Mouse brain cleared with CLARITY. Sample stained with Tyrosine hydroxylase (Cy3) and counterstained with Hoechst. 2 channels z-stack (ex. 405 nm + 561 nm), single side illumination.*

References:

[1] Hama H, et al., Nat. Neurosci. 14:1481 – 1488. (2011)

[2] Chung, K. et al. Nature 497, 332 – 337 (2013)

[3] Susaki, E. et al., Cell, 157, 726 – 739 (2014)

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