The Imaging of Three Dimensional Structures in Confocal Microscopy

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Optical microscopy and spectroscopy are key measuring techniques in scientific research. In these fields confocal microscopes are important instruments, combining the advantages of real 3D and spectroscopic imaging techniques. Confocal microscopy does not only offer a range of new applications but also creates new requirements of handling multidimensional data. This article describes some essential requirements a confocal 3D imaging system has to meet and explains the basic principles of visualisation needed to be able to interpret the recorded data.

3D Confocal Imaging

Confocal microscopy is a technology, which is used for a broad spectrum of scientific applications. It is the method of choice not only for recording the 3D shape of surfaces, but in particular for imaging 3D specimens which are to be investigated in vivo and therefore in a non-destructive and non-invasive way. Leica developed several types of confocal microscopes, each designed to meet the needs of a specific field of application. The Leica ICM 1000 (industrial confocal microscope) is typical of reflection microscopy used in material science to generate accurate mappings of surfaces. The Leica TCS SP2 (spectral confocal microsope) is typical of fluorescence microscopy. In the field of molecular biology it achieves what the variety of computer tomography techniques attains in medicine: volumetric 3D imaging. In fact, there are even more advantages. In fluorescence microscopy the recording of structural 3D data is directly coupled with spectroscopic properties of the specimen. Thanks to advances in biochemistry, it is possible to use fluorescent dyes to selectively mark literally any component within cells. This staining method permits the detection and localization of a wide range of macromolecules like actin molecules or sequences of DNA base pairs and therefore the acquisition of a wealth of spectral information from one – most of the time living – specimen. With the TCS SP Leica introduced a spectrophotometer, which guarantees flexibility and efficiency with all fluorescent dyes that conventional filter based systems cannot provide. It allows optimal 3D imaging of dyes with difficult spectral properties.

All Leica confocal microscopes (TCS NT, MP, SP, SP2, E and ICM 1000) are delivered with the new *Leica Confocal Software* (LCS), software tailored to three-dimensional and spectral imaging techniques. A schematic overview of the multi-tier architecture of the LCS is shown in figure 2.

Many of its features are especially designed for 3D and higher dimensional imaging. A 3D navigation feature, for example, has been created with a special "*Series Scan Overview*" (see Figure 3.). This overview displays the 3D partitioning of the specimen into sections while the image is being recorded and thus explains geometry and internal system states. In addition special visualizations are provided which open up completely new insights into the structure of the specimen or of processes over time. Other software features are adjusted to special application domains, like the analysis of physiological activities inside of living specimens or the DIN/ISO compliant analysis of material surfaces. Because of the large diversity of application fields, a - Visual Basic™ based - macro capability is incorporated into the software, which allows the user to adapt the system to any application field. This macro capability gives access to all software functions, from hardware control to visualization processes. The spectrophotometer, for example, is mapped as the *Spectrophotometer* object that routes commands to the hardware device (external devices layer) while the *ImageTool.Renderer* object enables all kinds of visualization processes. With this modular concept the system can easily be adapted to other applications, such as 3D imaging of physiological activity, 4D microscopy over time and 4D microscopy over spectral studies.

3D Rendering – Principle and Insights

Optical sectioning provides the basis for confocal three-dimensional imaging. An advantage of this technique is the reduction of the scanned probe volume and in particular the enhanced resolution. The recorded confocal data consists of sets of single images forming a volumetric stack. Compared to conventional light microscopy, the images contain more details. However, the problem is that human visual perception has difficulty in recognising three-dimensional structures from sectioned data. It is visualisation techniques that help building a mental map of the recorded object and launches a creative, cognitive process. Since this cognitive process is most effective when the user interactively creates several views of the object, very fast and interactive computer algorithms are incorporated into the LCS.

Reflection is useful for the characterisation of microstructures. We know its behaviour well from our macroscopic life. If something is in front, we cannot look at things behind it. These characteristics led to efficient algorithms based on models of surfaces as polygon patches. LCS uses this today's industry standard. A surface is searched in the volumetric image (segmentation) and used as a basis for the polygon model. Our systems allow the fully interactive manipulation of such scenes based on OpenGL™ technology (Figure 5).

A fluorescent object is transparent and segmentation is difficult to obtain. For this reason the LCS includes volume-rendering algorithms that do not need any kind of segmentation. These algorithms project a 3D volume onto a 2D screen. The basic principle is to examine and superimpose the

volume elements throughout all optical sections into the resulting image. The direction from which the volume is viewed can be free selected as well as the kind of projection to be used. The projection types differ in how the volume elements are evaluated. The maximum projection selects the brightest point on the way through the volume elements and displays it in the 2D image. This projection type shows the most prominent structures of a specimen. A real 3D visual cue is not achieved, because if you look from behind or from front will result in the same projection. The average projection calculates the mean value of all examined volume elements thus also including not prominent structures. This projection type visualises additional details, but also costs image dynamics. The transparent projection takes into account that the intensity of light decreases on the way through the volume. A real 3D visual cue can be obtained because structures in front are weighted differently than those behind. With an SFP projection (simulated fluorescence process algorithm) a fluorescence process is simulated and additional visual cues can be provided by means of an artificial shadow. This rendering method is the most realistic for imaging fluorescent specimens, in particular if they are marked with several fluorescent dyes and recorded at multiple wavelengths. Figure 6 provides an overview of different projection types.

Visual Cues and Steropsis

As an option additional visual cues are provided by animations and stereo images calculated by arbitrary projections. You may also create animations of stereo images. To generate animations the LCS looks at the specimen from multiple viewing angles defined by the user and calculate a movie. The calculated animation is compatible to the multimedia software included in the operating system.

Stereo rendering combines two projections, one projection for the right and one projection for the left eye. Disparity is introduced using different adaptable viewing angles (of approximately 6°) for the projections. The result image can be viewed with special stereo eyeglasses. The images shown here are red/green anaglyphs and can be viewed with the red/green eyeglasses included in this magazine (provided the observer does not suffer from red-green blindness). The eyeglasses work as a filter that projects the image onto the corresponding eye. Thus it is possible to see a 3D image, but colour information is lost on the way through red/green eyeglasses. The human brain fuses stereo images by exactly the same mechanism that enables 3D scene perception. This common mechanism of perception is named stereopsis. The exact biological mechanisms that cause stereopsis is still under research, but you may see the amazing effect by looking at the images through the glasses.

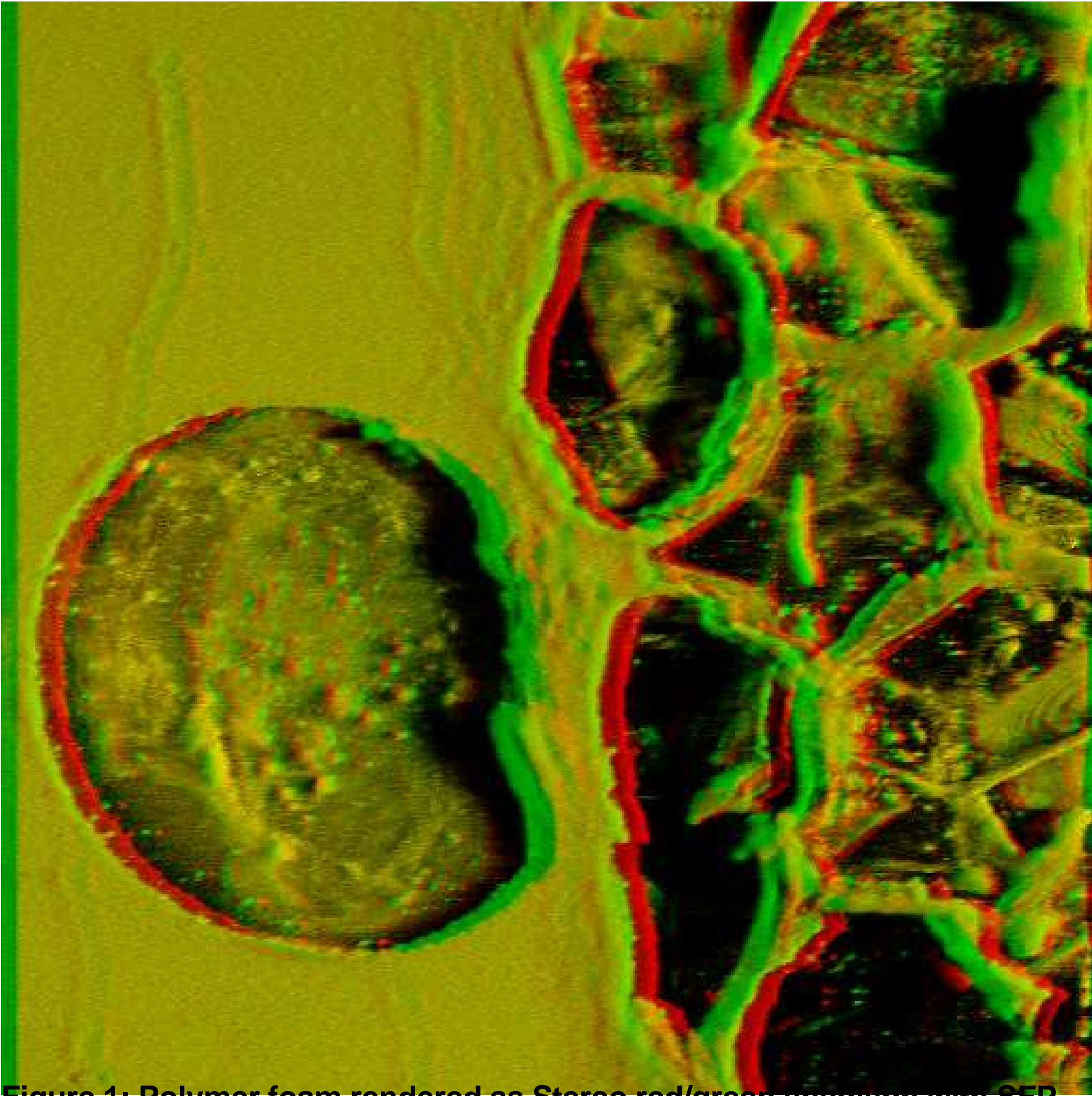

Figure 1: Polymer foam rendered as Stereo red/green anaglyph with SFP projection.

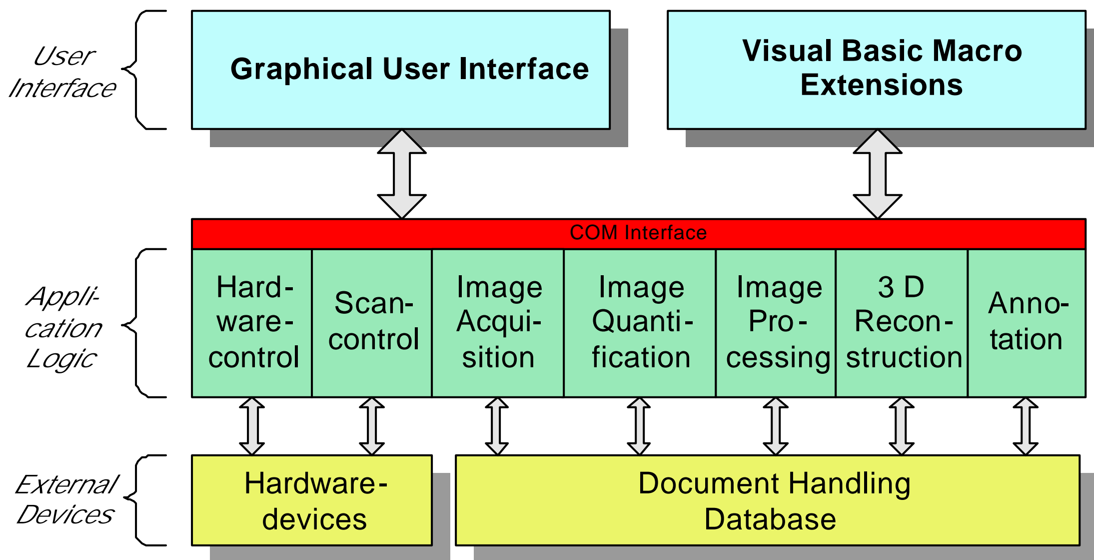

Figure 2: Overview architecture of Leica LCS:
All elements needed for 3D and multidimensional imaging are included in the architectural framework.

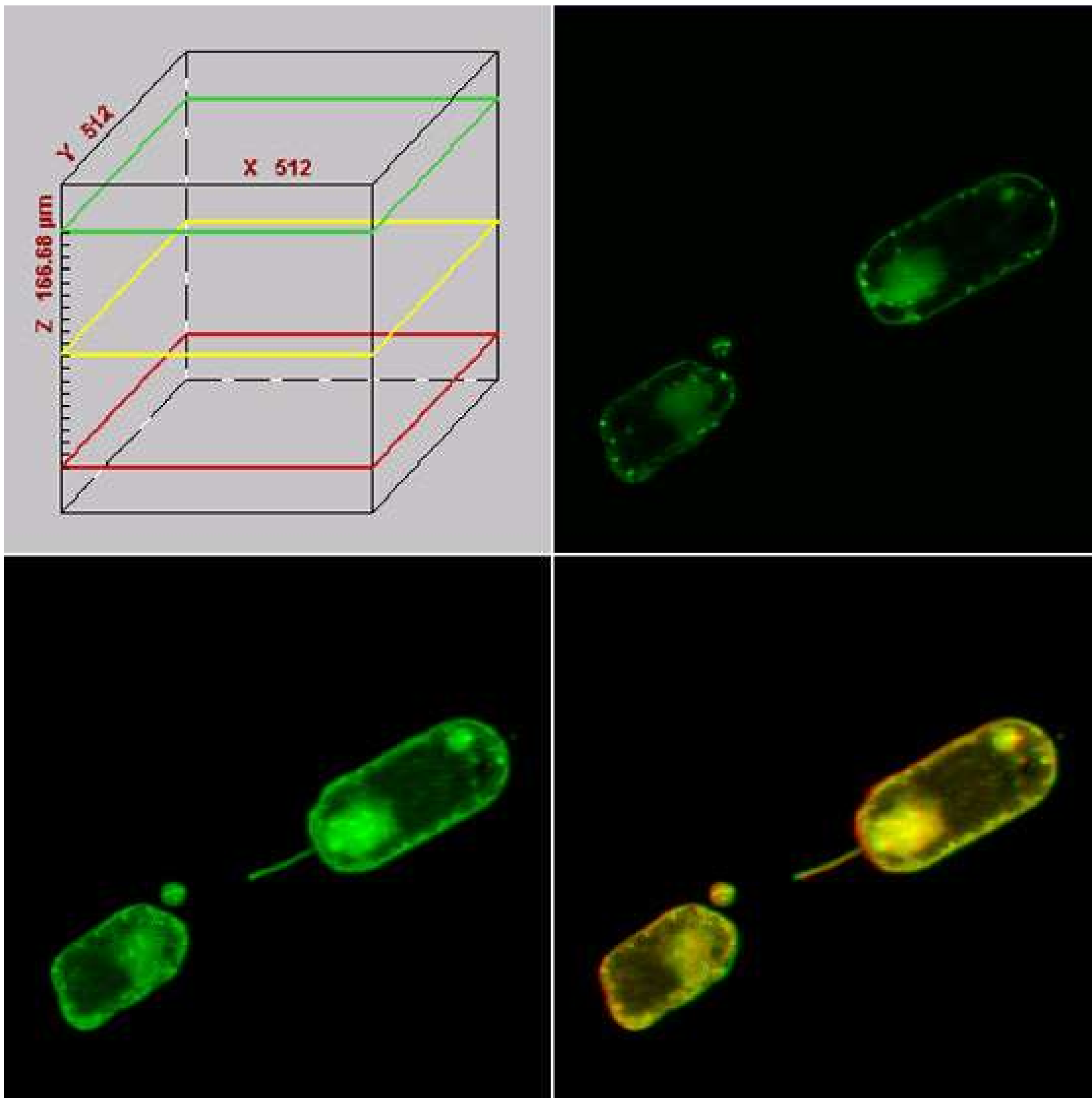

Figure 3: The way from confocal volumetric images to stereo object display. The specimen shown is a planctonic, centric seawater diatom (Ditylum brightwellii). Pane description from left top to bottom right: (a) Overview of the Scan Geometry (b) One Slice of the volume stack located at the yellow plane from pane a. (c) Transparent Projection of complete volumetric stack. This image is similar to viewing through a standard microscope. (d) Stereo Transparent Projection of volumetric stack displayed as red/green anaglyph.

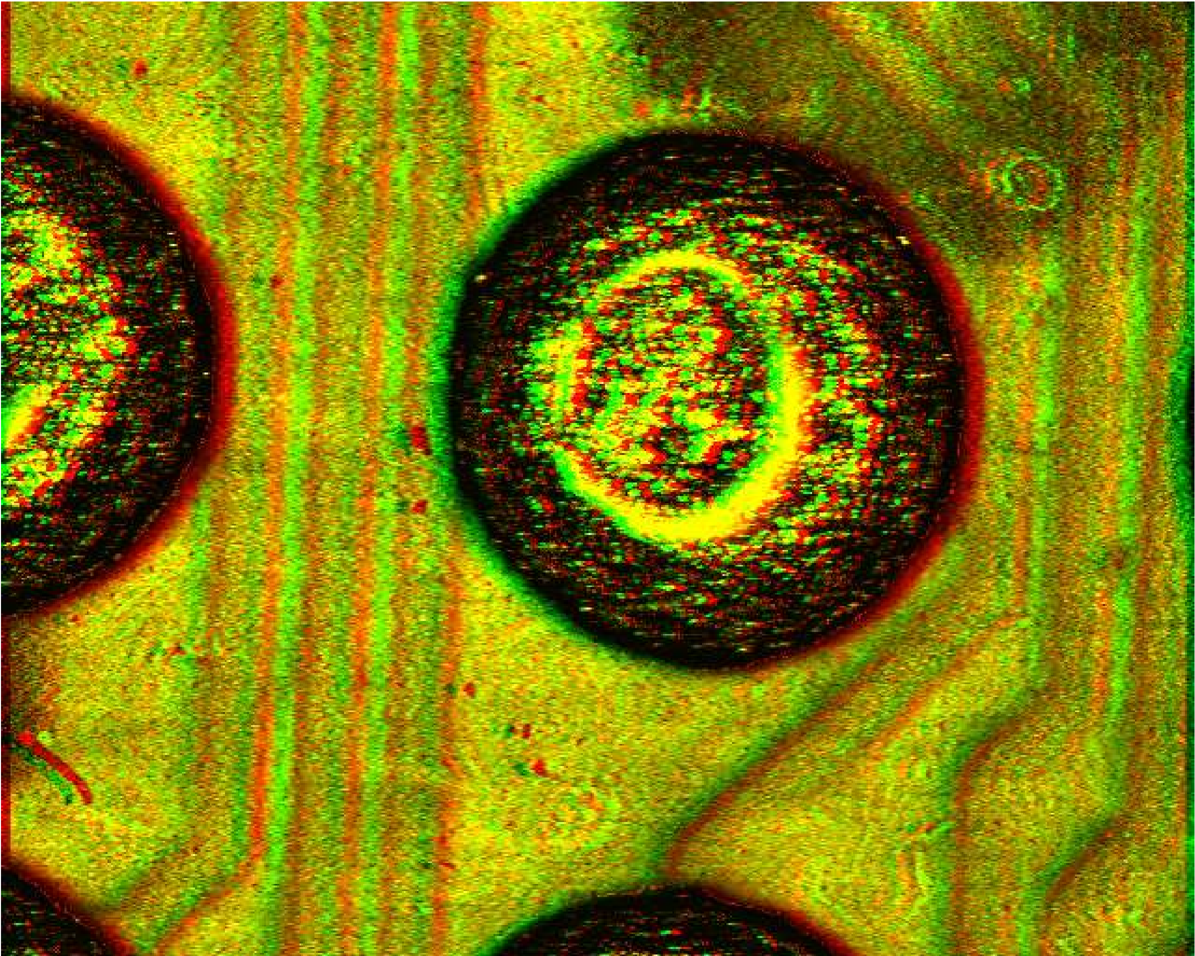

Figure 4: Red/green Anaglyph of a printed circuit board (PCB) with soldering (Maximum Projection).
Please notice the large height differences between soldering and the structure on the PCB.

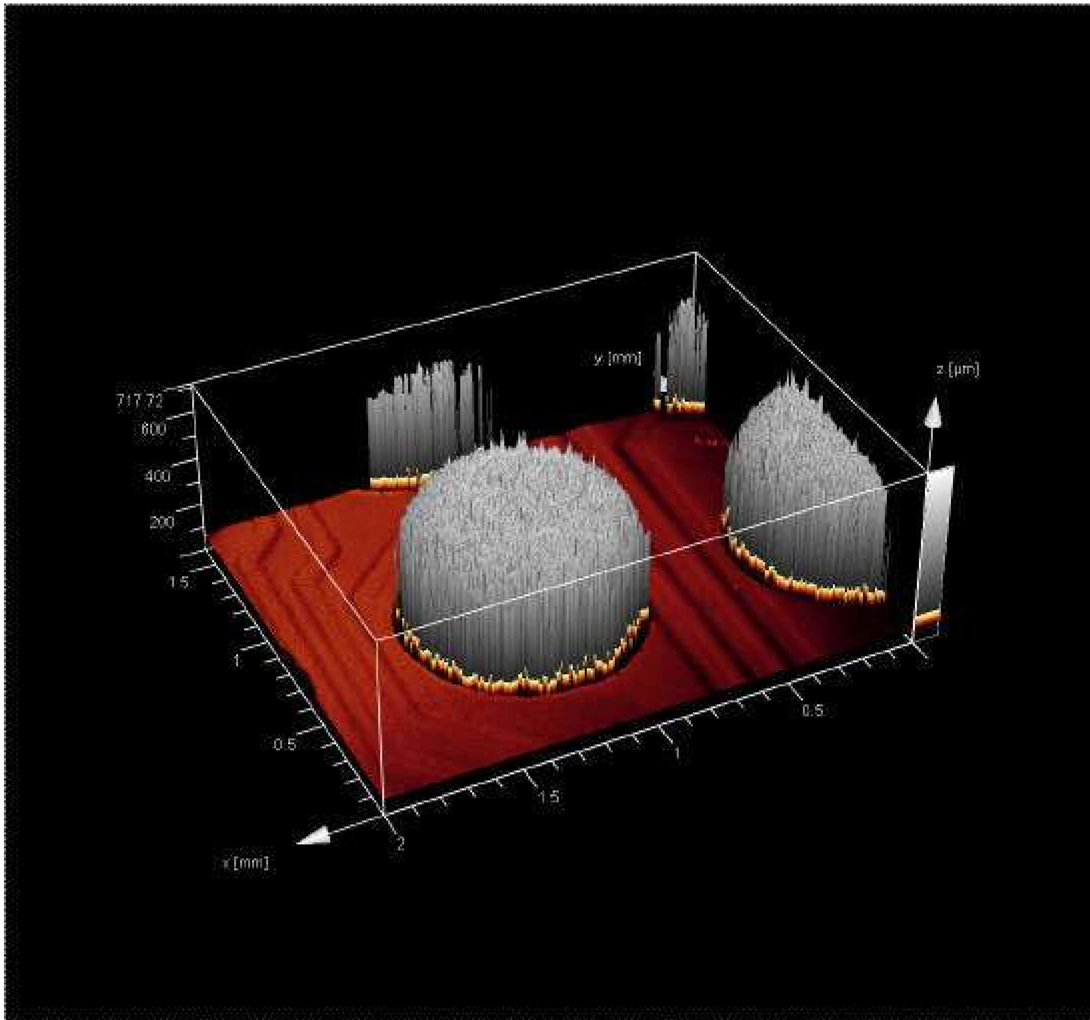

Figure 5: Surface visualization of topographic depth map of PCB with soldering.

Because of the large differences in height (high image dynamics), intensity level slicing has been used to visualize the details on the PCB

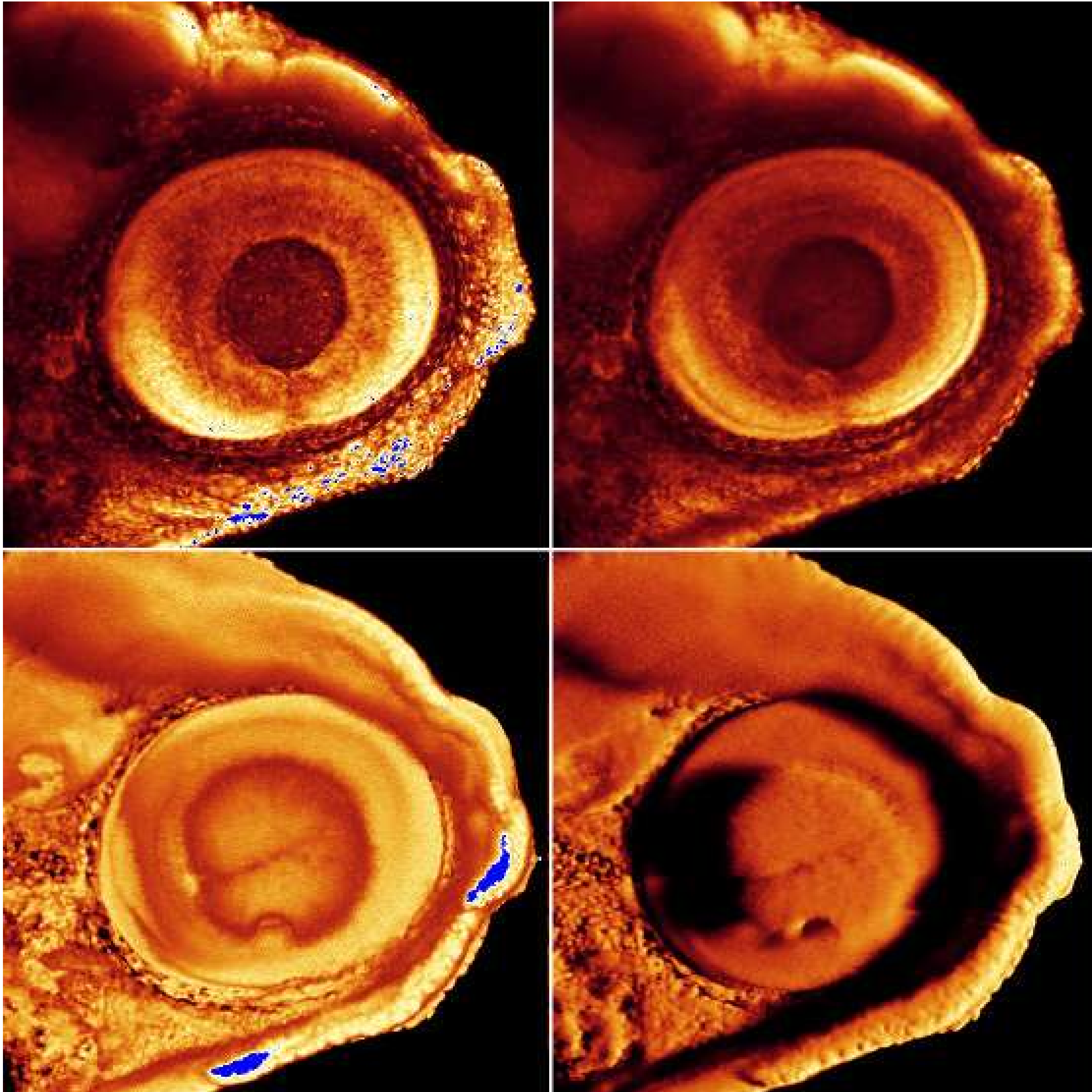

Figure 6: Overview of different projection methods.
The specimen is a zebrafish larva. The projection looks from the inside into the eye. Render method from left top to bottom right: (a) Maximum projection, (b) average projection, (d) transparent projection, (d) SFP projection

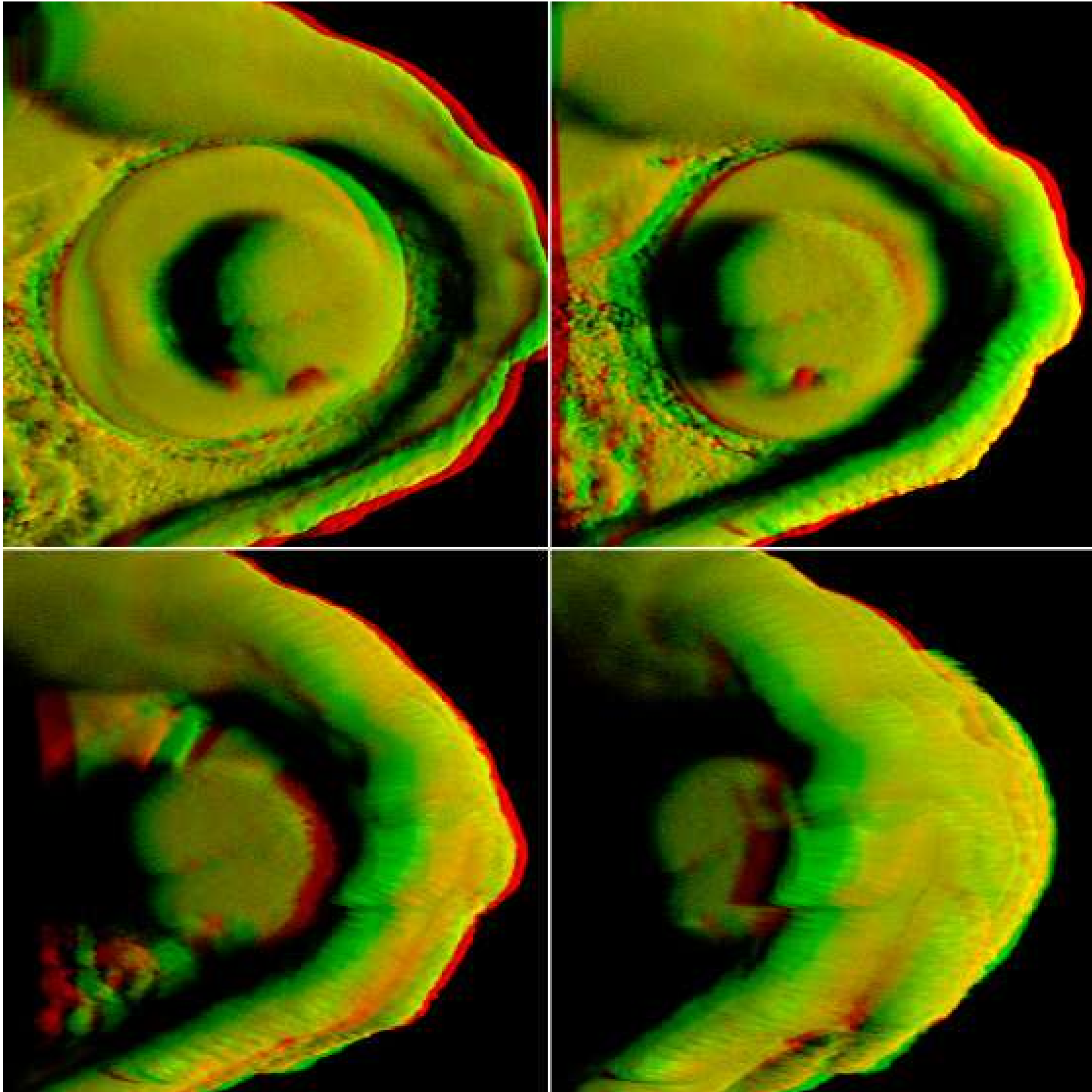

Figure 7: Single Images from an Animation (SFP) of the specimen from fig. 6. Slightly different viewer positions are simulated by rotation.