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User-oriented image processing throughout the changing times

BY WOLF MALKUSCH

IMAGE PROCESSING systems have been commercially available for more than 35 years.[1] While the first systems were hardware based and only offered a few algorithms and measurement parameters, they produced results with a speed that has yet to be reached with today's software-based systems. But the change from these pure hardware-based systems to today's use of software-based systems offers an enormous increase in flexibility and user friendliness. As a result, users of today can choose between various generic systems that they can implement as image analysis toolboxes to easily tailor their own solution for any application-specific imaging problems.

Quantitative FRET measurements: View and measure the invisible

Fluorescence resonance energy transfer (FRET) is a relatively new technique of measuring the distance between two protein molecules using different fluorescence markers. By combining modern image processing with light microscopy it is now possible via FRET to determine quantitative timing and spatial information about bindings and interactions of proteins, lipids, enzymes, DNA, and RNA in living cells. Various methods have been described in the literature that document how the FRET amount can be measured and corrected to represent absolute values.[2–5] These methods can be implemented quite easily in modern image processing systems (e.g., the KS 400) (**Carl Zeiss Vision GmbH,** Hallbergmoos, Germany).

For FRET experiments with mutants of the green fluorescent protein (GFP), for example, the blue or cyan GFP (CFP) and the yellow GFP (YFP) are used. Images are acquired with a fluorescence-capable, stable, integrating digital camera (for example, the Axiocam MRm, **Carl Zeiss Vision GmbH**) on a fully motorized fluorescence microscope (for example, the Axiovert 200 mot, **Carl Zeiss Vision GmbH,** *Figure 1*). The directly measured FRET image will be "corrected" by images from the CFP and YFP channel. FRET intensities are directly extracted from the false color image via scaling functions within the

Figure 1 *Cell Observer: the Axiovert 200 mot microscope with climate box, Axiocam HR digital camera, and Axiovision image processing system.*

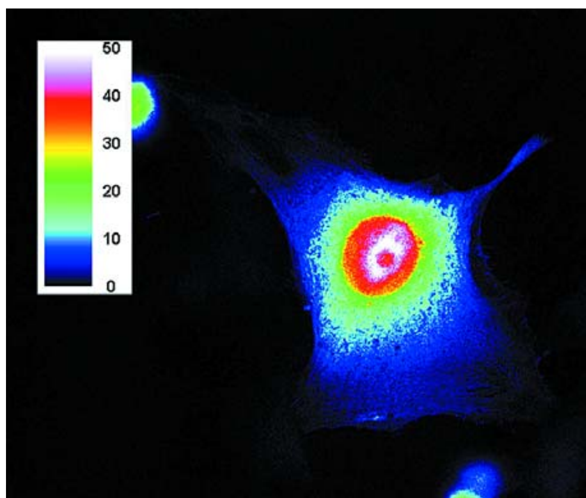

Figure 2 *FRET false color display. Fc = FRET – CFPkorr – YF - Pkorr. Cell specimen with cells marked with CFP only, YFP only, and CFP and CFP plus YFP, objective; 40×, 1.3 oil. Fc method by Youvan et al. (Ref. 4). Photo taken during the DKFZ Digital Microscopy course, Heidelberg, Germany, 2001.*

display overlay (*Figure 2*). Additionally, cell structures can be measured directly using variable measurement frames. Additional time-dependent measurements are

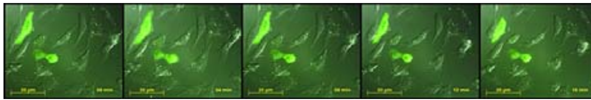

Figure 3 *Time-lapse sequence acquired using the Cell Observer. Living cells over 16 min, 1 image per min, combined differential interference contrast (DIC) and GFP image, objective 63×, 1.25 oil. Image sequences taken at the MDIBL Fluorescence Microscopy course, Bar Harbor, ME, 2001.*

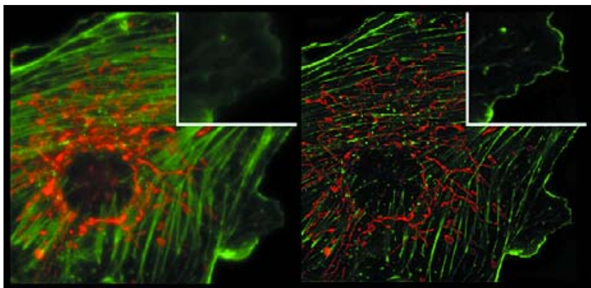

Figure 4 *Bovine epithelial cells, cytoskeleton fluorescein isothiocyanate (FITC), mitochondria rhodamine, objective 100×, 1.3 oil. a) original image and b) result of a 3-D iterative maximum likelihood deconvolution. The inserts show a detail in higher magnification.*

possible to quantify bleaching effects or fluorescence recovery processes.

Cell Observer: View life with new eyes

Apart from configurations with nearly unlimited possibilities, as offered by the generic systems, requirements now exist for the highest degree of automation to allow automated repetitive sequences in light microscopy. With the revolution in fully integrated motorized functionality of the microscope hardware, coupled with the migration from wet film to digital imaging, new possibilities have emerged in the field of live cell imaging.

As a consequence, images are no longer restricted to two dimensions. Only a short time ago we were happy to acquire, display, and evaluate three-dimensional images with relatively little effort.[6] Today, systems such as the Cell Observer (Figure 1) are available that are able to automatically acquire 4-D, 5-D, and even 6-D images. In addition to 2-D images acquired as a 3-D stack, multichannel fluorescence can also be captured separately (4-D). The whole procedure can be repeated in multiple time frames (5-D, *Figure 3*). If the system is equipped with a motor stage, then the complete sequence can be performed at various predefined positions (6-D).[7]

3-D deconvolution: Playing a trick on the resolution limit

All of these costly methods have been enabled by the enormous progress in computer technology. Extremely high storage capacities of more than 1 GB RAM memory and clock frequencies far beyond 1 GHz enable most complex computation processes to be performed in acceptable time frames not even dreamed of only a few years ago.

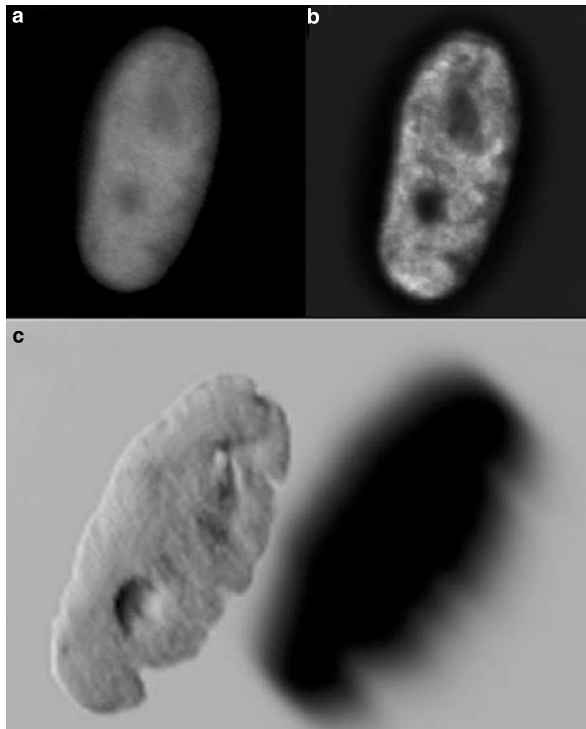

Figure 5 *Cell nucleus, red GFP, objective 63×, 1.25 oil. a) original image, b) result of an iterative 3-D deconvolution, c) 3-D reconstruction from the 3-D image stack of the nucleus after 3-D deconvolution in shading image mode. Images taken during the DKFZ Digital Microscopy course, Heidelberg, Germany, 2001.*

One of the oldest goals in the field of light microscopy was to use mathematical algorithms to increase optical resolution by eliminating the haze and blur caused by out-of-focus information. Computational formulas for deconvolution were first described in the 1930s.[8] Until recently, it was not possible to implement these algorithms in cost-effective computers that would perform all the necessary calculations within an acceptable time frame.

A prerequisite is a 3-D stack of images, acquired with a stable digital camera on a highly precise fluorescence microscope (e.g., with a Cell Observer system). By optimizing all aspects of image input (e.g., light source, filters, objective correction, digital camera resolution, etc.),[1] outstanding improvements in final output performance can be achieved (*Figure 4*).

Conclusion

Today there are a wide range of 3-D deconvolution methods in use. The “nearest neighbor” and the “inverse filter” groups produce clear image improvements

with relatively low efforts. But neither of these groups are capable of improving resolution. An increase in resolution can only be achieved by the so-called "iterative" 3-D deconvolution methods (e.g., "maximum likelihood" or "Jansson/van Cittert" methods). On the basis of these processed image stacks with removed blur, 3-D reconstructions can be produced that clearly show the spatial arrangement of the structures (*Figure 5*). With the implementation of a host of new 3-D animations, additional details can be easily detected.

References

1. Bauch H. System for cultivating and digitally documenting living cells or tissue. Biofor Internat 2002; 1:18–19.
2. Gordon GW, Berry G, Liang XH, Levine B, Herman B. Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. Biophys J 1998; 74:2702–13.
3. Siegel RM, Chan FK, Zacharias DA, et al. Measurement of molecular interactions in living cells by fluorescence resonance energy transfer between variants of green fluorescent protein. Science's stke (Signal Transduction Knowledge Environment) 2000:1–6.
4. Youvan DC, Silva CM, Bylina EJ, Coleman WJ, Dilworth MR, Yang MM. Calibration of fluorescence resonance energy transfer in microscopy using genetically engineered GFP derivates on nickel chelating beads. Biotechnology 1997; 3:1–18.
5. Xia Z, Liu Y. Reliable and global measurement of fluorescence resonance energy transfer using fluorescence microscopes. Biophys J 2001; 81:2395–2402.
6. Malkusch W. Trends in quantitative image analysis. Biomed Prod 1998; 6:18–20.
7. Bauch H. System for cultivating and digitally documenting living cells or tissue. Biofor Internat 2001; 1:18–19.
8. van Cittert PH. The influence of bandwidth on the intensity distribution in spectral lines. Zeitschrift für Physik 1930; 65: 298–308.

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