Reproduced from Microscopy and Analysis ©2015 John Wiley and Sons Ltd

The ATUMtome for automated serial sectioning and 3-D imaging

Paul Webster[1], Dave Bentley[2] and Jonelle Kearney[2]

1 Oak Crest Institute of Science, Pasadena, California, USA; 2 RMC Products by Boeckeler Instruments, Tucson, Arizona, USA

INTRODUCTION

Serial sectioning has always been a powerful approach for microscopists to obtain three-dimensional (3-D) information from larger volumes of samples. The information obtained from serial thin sections is digitally reconstructed to produce models of what the original sectioned tissue may have looked like. This approach is especially powerful for electron microscopy where fine ultrastructural connections of subcellular compartments can be revealed in 3-D.

With the introduction of array tomography (Micheva & Smith, 2007), serial sections collected onto glass slides (Micheva & Smith, 2007) or silicon wafers (Horstmann, et al., 2012) can be immunolabeled and imaged by epifluorescent microscopy to obtain 3-D information on antigen distribution. Imaging the same serial sections in a field emission scanning electron microscope using backscattered or secondary electrons produce a stack of images with higher resolution than can be obtained by light microscopy. The ultrastructure revealed in the SEM images makes it possible to correlate antigen distribution (from light microscopy) with high-resolution images of the reference space. Structures are revealed that do not label with antibodies and are thus invisible under epi-fluorescence microscopy. Most importantly, the Z-axis of the 3-D reconstructions, determined by the section thickness, can be known and easily controlled.

The ability to obtain serial sections for 3-D imaging recently took a giant step forward with the introduction of the Automatic Tape-collecting UltraMicrotome, or ATUMtome (Schalek, et al., 2011). Now it is possible for anyone with basic ultramicrotomy skills to collect thousands of serial sections and reconstruct sectioned volumes in three dimensions.

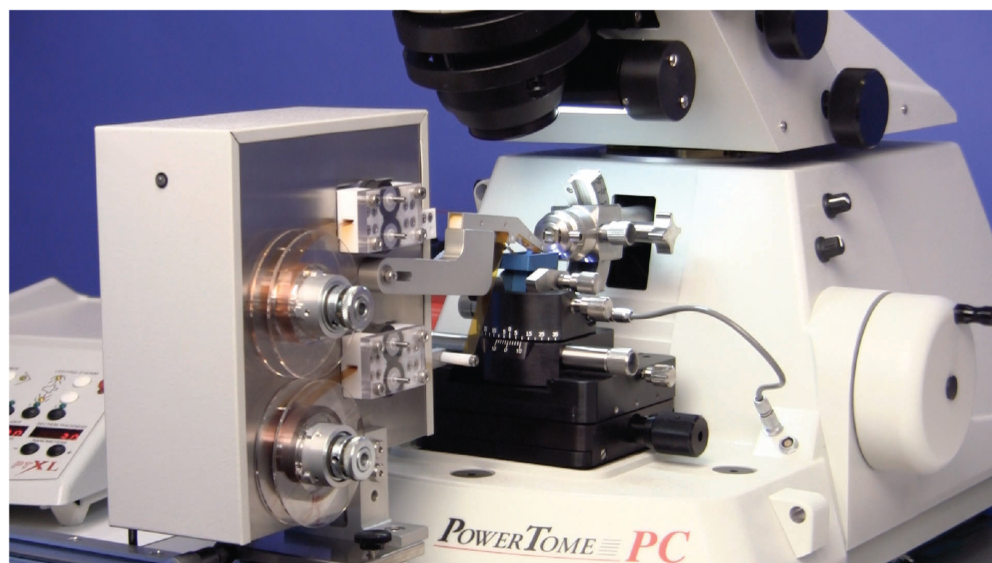


FIGURE 1 The ATUMtome, consisting of a tape collection unit (the "ATUM") attached to the front of an ultramicrotome (the "tome"). The ATUM dispenses tape from a reel into the water trough of the diamond knife via a specially designed arm. The tape moves through the water surface and is taken back onto a second reel located above the first reel.

RESULTS

ATUMTOME FOR LIGHT MICROSCOPY

The ATUMtome is a combined ultramicrotome and tape collection device marketed as a single unit (Figure 1). The ultramicrotome (the "tome"), a familiar tool for electron microscopists, is designed to cut reproducibly thin sections of resin-embedded material. The tape collection device, or ATUM (Schalek, et al., 2011), developed to collect the sections produced by the ultramicrotome, attaches to the front of the ultramicrotome.

Serial sections of the resin-embedded material are cut by a diamond knife and floated onto the water surface of the knife trough with the tape dispenser placed close to the knife edge (Figure 2). The sections are then collected in sequential order from the water surface by a ribbon of tape dispensed by the ATUM (Figure 3). Tape with attached sections is collected onto a spool where it can be removed and mounted onto a flat substrate for immunolabeling. Typical substrates include glass slides or silicon

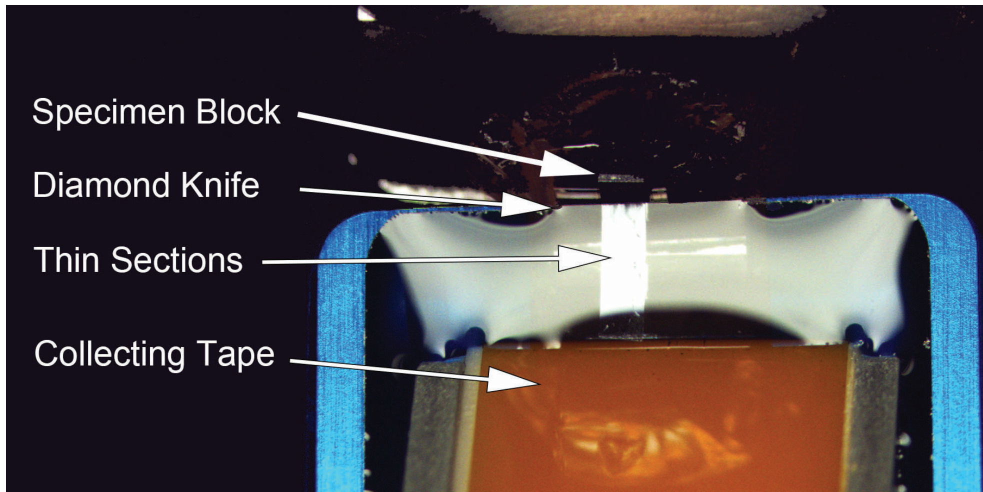


FIGURE 2 Close up of the diamond knife with the collecting tape placed close to the knife edge and partially submerged in the water. A specimen block of resin-embedded material is drawn past the edge of a diamond knife to cut thin sections. The sections float on a water surface maintained in a trough around the knife and are pushed to the collection tape by subsequent sections. A tube on the left side of the diamond knife is connected to an automated water dispenser that maintains a constant water level in the knife trough.

FIGURE 3 The collecting tape removes the sections from the water surface where they dry and are rolled onto a spool.

wafers (Figure 4).

Flat mounted tape with attached sections can either be imaged directly (if the sectioned material contains endogenous fluorescent probes) or the sections can be immunolabeled with primary antibodies and secondary fluorescently labeled visualization probes. Fluorescent sections are then imaged under epifluorescent illumination.

Serially sectioned, ultrathin resin sections have many advantages over confocal scanning laser microscopy (CLSM). The Z-axis resolution by CLSM is at best 0.48 µm (Hell, et al., 1993), which is insufficient to image small structures such as vesicles or synapses. With thin sections, the Z-axis resolution is limited only by the section thickness (down to 30 nm) so that fine details within cells and tissues can be included in 3-D reconstructions. Immunolabeling of thin sections of resin-embedded material is usually restricted to the section surface (Schwarz & Humbel, 2007), a phenomenon that coincidentally improves resolution in the X-Y axis too.

IMMUNOLABELING CAN BE REPEATED

Immunoreagents applied to the serial sections can be eluted and thus it is possible for a different combination of antibodies and visualization probes to be repeatedly applied. In one study, nine consecutive double labeling experiments were carried out on serial sections of LR White embedded material (Micheva & Smith, 2007).

Immunoreagents are eluted from resin sections by exposure to a solution of 0.15M potassium permanganate in 0.01N sulfuric acid for 90 sec (Tramu, et al., 1978), or by treating the sections for 20 min with 0.2 M NaOH and 0.02% SDS in distilled water (Busse & Smith, 2013).

IMAGING FLUORESCENTLY TAGGED PROTEINS

Arrays of serially sectioned, resin embedded biological material have been used to locate fluorescently-tagged endogenous markers. Until recently, the challenge to this approach has been the ability to preserve fluorescence through all the processing steps necessary for resin embedding. Gibson and team (Gibson, et al., 2014) recently published a detailed study of the effects of chemicals used to prepare specimens for resin embedding. The authors of that study offer useful strategies for preserving a wide range of endogenous fluorescent markers after resin embedding. However, preservation of GFP fluorescence in cells may be as simple as rapidly freeze substituting high pressure frozen cells (McDonald & Webb, 2011) and embedding in Lowicryl HM20 resin (Peddie, et al., 2014).

Alternative approaches to detecting fluorescently tagged endogenous markers in resin sections have included the use of antibodies to the fluorescent marker (Oberti, et al., 2010), reactivation of quenched fluorescence (Xiong, et al., 2014), embedding in less traditional resins (Yang, et al., 2013), and photo-oxidation (Ellisman, et al., 2012; Grabenbauer, 2012).

ATUMTOME FOR ELECTRON MICROSCOPY

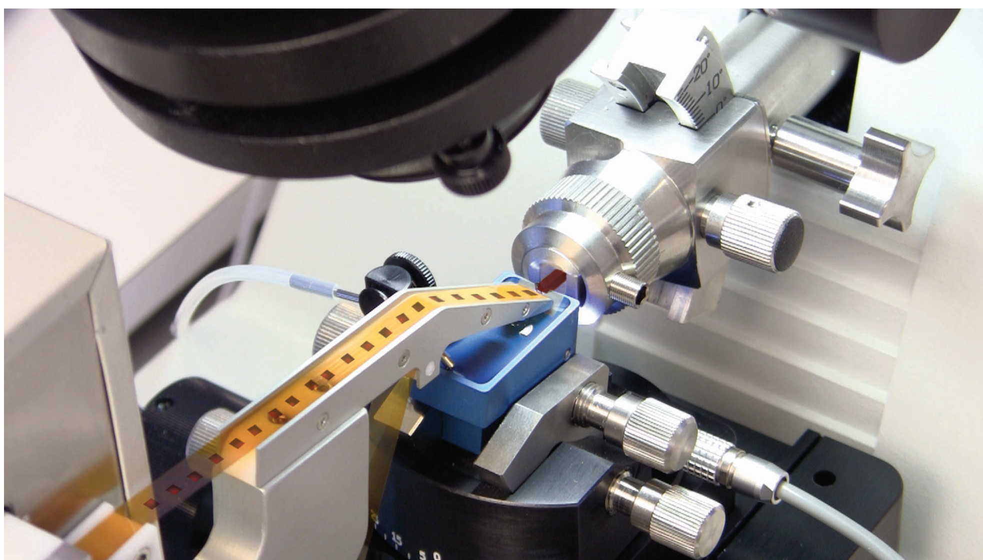
The serial sections, mounted on tape and on silicon wafers, can be contrasted with uranyl acetate and lead citrate and imaged in a field emission scanning electron microscope. Imaged using a back scattering or secondary electron detector and then inverted digitally, the microscope produces images comparable to those obtained using a transmission electron microscope. The FESEM is able to image tissue sections on Kapton tape or glass (Tapia, et al., 2012).

Some users apply image contrast before resin embedding by treating biological specimens with multiple rounds of heavy metal solutions and mordants (Tapia, et al., 2012). These treatments increase specimen contrast and thus subsequent segmentation studies, assist in SEM imaging by improving specimen conductivity, and avoid the disadvantage of post-section stain precipitation.

CORRELATIVE LIGHT AND ELECTRON MICROSCOPY STUDIES

Resin embedding for immunolabeling and examination under epifluorescent illumination has been a staple technique for CLEM approaches (Schwarz & Humbel, 2007; Schwarz & Humbel, 2014). However, most early applications used single, sequential sections to compare light microscopy with electron microscopy. One section was labeled with fluorescent probes and one section was examined by transmission electron microscopy. Information was usually collected in two dimensions, which limited the ability to determine associations between multiple structures through the specimen volume.

The ATUMtome is able to automatically section through large volumes of resin embedded material, collecting the serial sections in sequential order onto a tape for imaging



AM

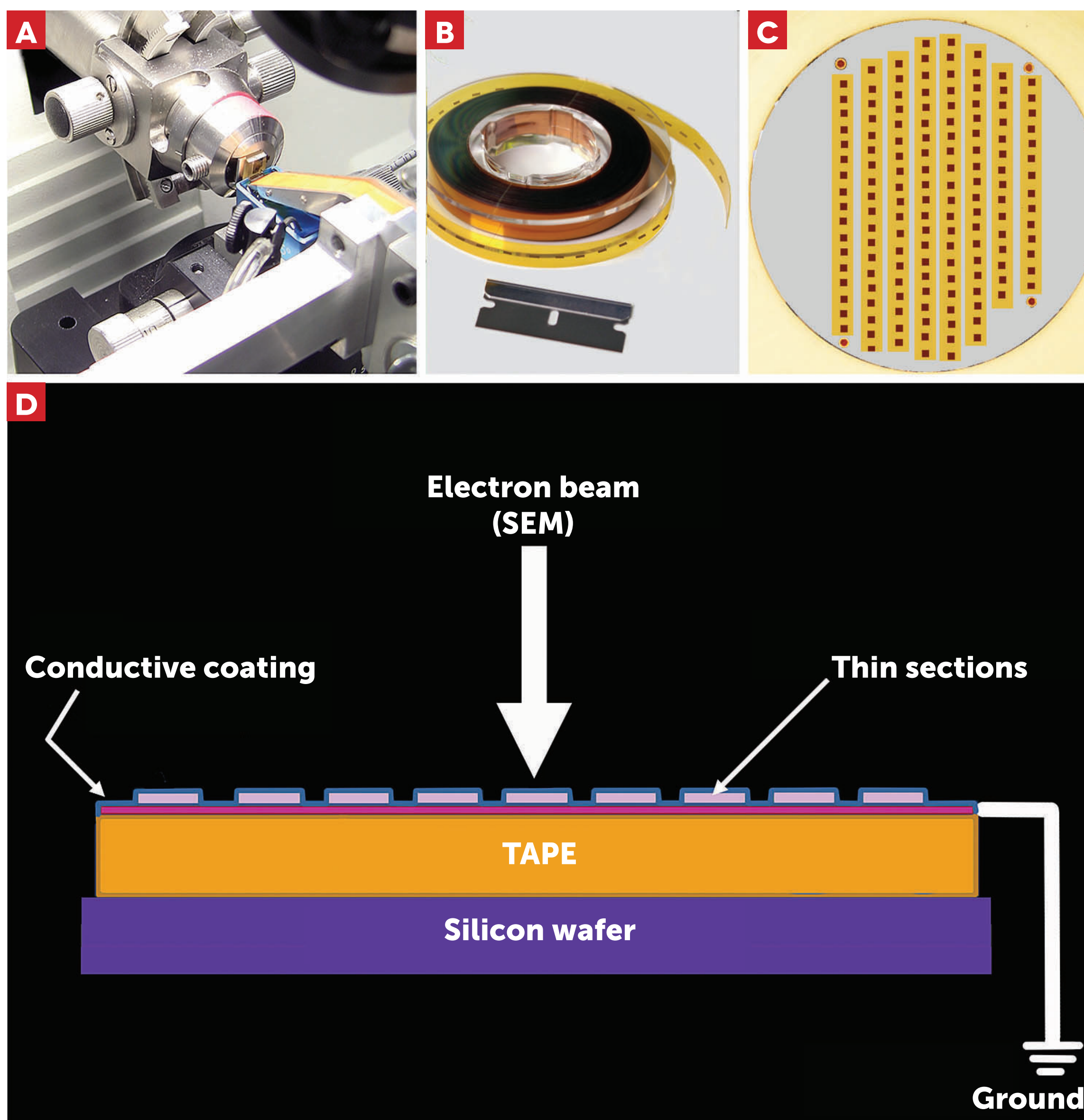


FIGURE 4 Section collection is performed automatically using the ATUMtome (A). The tape with sections attached is removed from the spool and cut into small strips (B) and attached to a 4" silicon wafer (C). This is an illustration of how the strips of tape are mounted onto the silicon wafer, with metal specimen grids placed at the corners of the wafer. The grids act as fiduciary marks for automated image collection (Hayworth, et al., 2014). The sections on the silicon wafer can be imaged using epifluorescent illumination (not shown), or with a scanning electron microscope (D). Carbon coating is present below the sections for SE imaging. For BSE imaging carbon is deposited over the sections.

by light microscopy. Sections can be labeled multiple times with specific antibodies, and imaged again in the scanning electron microscope to obtain high-resolution specimen detail.

Images collected in the scanning electron microscope can be combined with images of the same regions collected using epifluorescent illumination to produce 3-dimesional maps of protein distributions though cells or tissues.

SUMMARY AND CONCLUSIONS

Ultramicrotomy using an ATUMtome is a relatively easy approach for collecting large numbers of serial sections in ordered arrays. Attaching the collected sections onto silicon wafers and immunolabeling the sections is also relatively easy. However, collecting images of the labeled sections requires access to a light microscope with epifluorescent illumination that is able to hold silicon wafers. For imaging ultrastructure a field emission scanning electron microscope (FESEM) is required. The basic requirements of the FESEM are that it must have a detector for collecting backscattered electrons, in-lens detectors, and a specimen chamber large enough to handle silicon wafers. Tracking software that can locate the identical regions imaged by light microscopy would be a bonus feature on the FESEM.

The 3-D reconstruction software packages available (both free and commercial) must eventually be able to handle the large datasets generated from the potentially thousands of sections that can be imaged.

The ATUMtome is now being marketed by RMC Products by Boeckeler Instruments in Tucson, Ariz., under license from Harvard University, where the ATUM part of the instrument was developed by a team of neuroscientists under the leadership of Professor Jeff Lichtman.

The ATUMtome was introduced by RMC to the research community at the Neuroscience 2014 meeting held in Washington D.C., where it was well received for its roles in array tomography of large volumes, improved Z-resolution over confocal microscopy, 3-D reconstruction at the electron microscope level, and correlative light and electron microscopy (CLEM).

REFERENCES

1 Busse, B. & Smith, S. (2013). *Automated analysis of a diverse synapse population. PLoS Comput Biol* 9(3), e1002976.
2 Ellisman, M.H., Deerinck, T.J., Shu, X. & Sosinsky, G.E. (2012). *Picking faces out of a crowd: genetic labels for identification of proteins in correlated light and electron microscopy imaging. Methods Cell Biol* 111, 139-55.

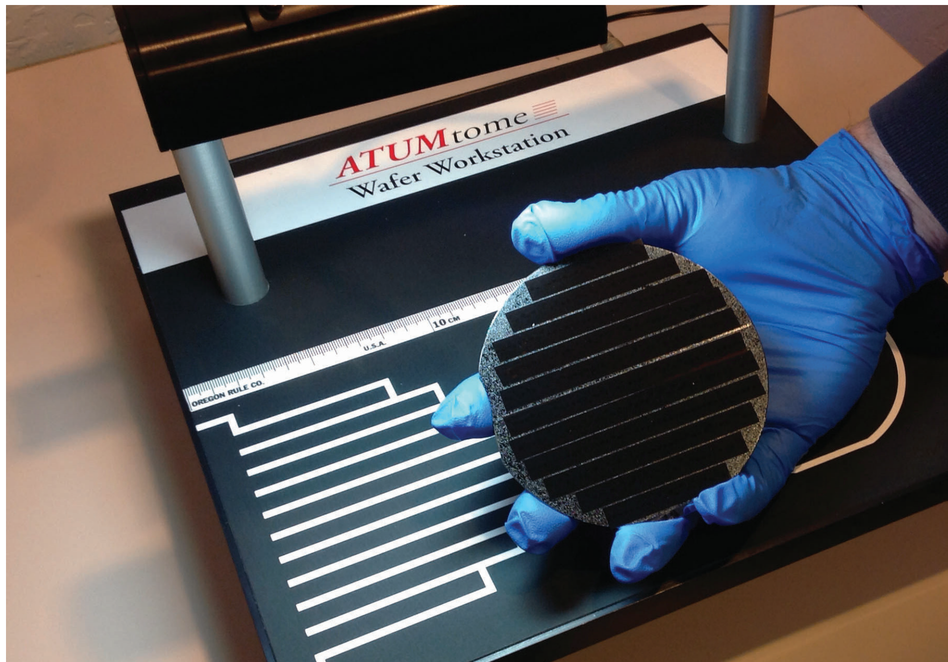


FIGURE 5
Ultrathin serial sections collected on Kapton tape and mounted to a silicon wafer are ready for imaging. Now it's possible for anyone with basic ultramicrotomy skills to collect thousands of sections. The resulting images could be used to reconstruct volumes in three dimensions. Smaller numbers of thin sections on tape can also be mounted onto glass microscope slides for examination.

3 Gibson, K.H., Vorkel, D., Meissner, J. & Verbavatz, J.M. (2014). *Fluorescing the electron: strategies in correlative experimental design. Methods Cell Biol* 124, 23-54.

4 Grabenbauer, M. (2012). *Correlative light and electron microscopy of GFP. Methods Cell Biol* 111, 117-38.

5 Hayworth, K.J., Morgan, J.L., Schalek, R., Berger, D.R., Hildebrand, D.G. & Lichtman, J.W. (2014). *Imaging ATUM ultrathin section libraries with WaferMapper: a multi-scale approach to EM reconstruction of neural circuits. Front Neural Circuits* 8, 68.

6 Hell, S., Reiner, G., Cremer, C. & Stelzer, E.H.K. (1993). *Aberrations in confocal fluorescence microscopy induced by mismatches in refractive index Journal of Microscopy* 169(3), 391-405.

7 Horstmann, H., Korber, C., Satzler, K., Aydin, D. & Kuner, T. (2012). *Serial section scanning electron microscopy (S3EM) on silicon wafers for ultra-structural volume imaging of cells and tissues. PLoS One* 7(4), e35172.

8 McDonald, K.L. & Webb, R.I. (2011). *Freeze substitution in 3 hours or less. J Microsc* 243(3), 227-33.

9 Micheva, K.D. & Smith, S.J. (2007). *Array tomography: a new tool for imaging the molecular architecture and ultrastructure of neural circuits. Neuron* 55(1), 25-36.

10 Oberti, D., Kirschmann, M.A. & Hahnloser, R.H. (2010). *Correlative microscopy of densely labeled projection neurons using neural tracers. Front Neuroanat* 4, 24.

11 Peddie, C.J., Liv, N., Hoogenboom, J.P. & Collinson, L.M. (2014). *Integrated light and scanning electron microscopy of GFP-expressing cells. Methods Cell Biol* 124, 363-89.

12 Schalek, R., Kasthuri, N., Hayworth, K., Berger, D., Tapia, J.C., Morgan, J.L., Turaga, S.C., Fagerholm, E., Seung, H.S. & Lichtman, J.W. (2011). *Development of high-throughput, high-resolution 3D reconstruction of large volume biological tissue using automated tape collection ultramicrotomy and scanning electron microscopy. Microsc. Microanal* 17 (Suppl. 2), 966-967.

13 Schwarz, H. & Humbel, B.M. (2007). *Correlative light and electron microscopy using immunolabeled resin sections. Methods Mol Biol* 369, 229-56.

14 Schwarz, H. & Humbel, B.M. (2014). *Correlative light and electron microscopy using immunolabeled sections. Methods Mol Biol* 1117, 559-92.

15 Tapia, J.C., Kasthuri, N., Hayworth, K.J., Schalek, R., Lichtman, J.W., Smith, S.J. & Buchanan, J. (2012). *High-contrast en bloc staining of neuronal tissue for field emission scanning electron microscopy. Nat Protoc* 7(2), 193-206.

16 Tramu, G., Pillez, A. & Leonardelli, J. (1978). *An efficient method of antibody elution for the successive or simultaneous localization of two antigens by immunocytochemistry. J Histochem Cytochem* 26(4), 322-4.

17 Xiong, H., Zhou, Z., Zhu, M., Lv, X., Li, A., Li, S., Li, L., Yang, T., Wang, S., Yang, Z., Xu, T., Luo, Q., Gong, H. & Zeng, S. (2014). *Chemical reactivation of quenched fluorescent protein molecules enables resin-embedded fluorescence microimaging. Nat Commun* 5, 3992.

18 Yang, Z., Hu, B., Zhang, Y., Luo, Q. & Gong, H. (2013). *Development of a plastic embedding method for large-volume and fluorescent-protein-expressing tissues. PLoS One* 8(4), e60877.

BIOGRAPHY

Paul Webster obtained his Ph.D. in the United Kingdom and is currently studying how bacterial biofilms form within the context of human health. He is a senior faculty member at the Oak Crest Institute of Science in Pasadena, California, an innovative chemistry research and education center that provides community college and high school students with first hand experience of scientific research in an academic environment. The Institute serves as a catalyst within the local community to increase science literacy and awareness of basic and applied research.

ABSTRACT

The progress in correlative light and electron microscopy (CLEM) has recently take a great step forward with the introduction of a device that attaches to an ultramicrotome and collects thin sections of resin-embedded material in sequential order. The ATUMtome is a logical extension of the array tomography serial sectioning approach to imaging biological material that was recently introduced. Whereas array tomography manually collected limited numbers of serial sections onto glass slides or silicon strips, the ATUMtome is able to automatically collect thousands of sections onto a tape for imaging by light or electron microscopy. Originally designed for studying neuronal connections in the brain, the ATUMtome can be used to image large volumes of any organ or tissue where 3-dimensional relationships between structures are important.

ACKNOWLEDGEMENTS

All images are courtesy of Boeckeler Instruments Inc.

CORRESPONDING AUTHOR DETAILS

Paul Webster, Ph.D
Oak Crest Institute of Science
2275 Foothill Blvd,
Pasadena, CA 91107 USA
pwebster@usc.edu

Dave Bentley, M.S. and Jonelle Kearney
RMC Products by Boeckeler Instruments
4650 S. Butterfield Drive
Tucson, AZ 85714 USA

Microscopy and Analysis 29(2): 19-23 (AM), March 2015