Chapter 18
Advanced 3D Optical Microscopy in ENS Research

Pieter Vanden Berghe

Introduction

Microscopic techniques are among the few approaches that have survived the test of time. Being invented half way the seventeenth century by Antonie van Leeuwenhoek and Robert Hooke, these techniques are still essential in modern biomedical labs. One of the most important aspects in microscopy is the search to improve resolution as well as the contrast between the item of interest and the background. Different contrast techniques have been invented (phase contrast, differential interference contrast, Hoffmann modulation,…) to make sure that (sub)cellular structures could be identified using light. These techniques have been essential in ENS research since all sharp electrode recordings were made on setups with this type of microscopy approach (Hirst and McKirdy 1975; Wood 1989). Apart from its use in electrical recordings, microscopy has been instrumental in the identification of subpopulations of cells in the ENS, using a variety of staining methods: silver impregnation, neurobiotin injections and antibody labeling.

A significant step forward in the use of microscopy was the introduction of fluorescence approaches. Due to the fact that the intense excitation light is now filtered away from the longer wavelength emission light, the contrast can be improved drastically. The development of different color fluorescent probes attached to selective antibodies has made it possible to identify subpopulations of enteric neurons in a variety of species (Costa et al. 1996; Furness 2000).

Another important impetus to the use of microscopy was the discovery and isolation of the green fluorescent protein (GFP) from the jellyfish Aequorea Victoria

On the occasion of the symposium "The ENS, 30 year later, Adelaide 2014"

P. Vanden Berghe (✉)
Lab for Enteric NeuroScience (LENS), TARGID, University of Leuven,
701, Herestraat 49, Leuven, Belgium
e-mail: Pieter.VandenBerghe@med.kuleuven.be

S. Brierley, M. Costa (eds.), *The Enteric Nervous System*, Advances
in Experimental Medicine and Biology 891, DOI 10.1007/978-3-319-27592-5_18

(Prasher 1995). In the last two decades different variants of this and other coral based fluorescent molecules have complemented the toolkit of the biomedical researcher. By analogy with the classic chemical ion sensors (Fura2, Fluo4 et al.), fluorescent proteins have been mutated and fused to become functional sensors to report pH (e.g. synaptopHluorin) or cytosolic Ca^{2+} concentrations (e.g. GCaMP). This approach has the advantage that, rather than having all cell layers contribute to the signal, only specific enteric glia or neurons will express the reporter, which expands the accuracy with which activity can be measured in a single cell (Boesmans et al. 2013; for review see Boesmans et al. 2015).

Apart from these biological developments, also optical, electronic and computational methodology has improved significantly, which permits to achieve higher resolution, more sensitive and faster recordings. In this paper a number of current developments in microscopy are highlighted in the context of ongoing enteric nervous system research.

3-Dimensional Imaging and Volume Reconstructions

Confocal imaging is an established technique that can be used to optically section fluorescently labeled samples. It can be used to create high quality two dimensional (2D) images because out of focus light is efficiently removed. Apart from generating higher quality 2D images, the three dimensional (3D) image stacks can also be used to accurately determine cellular shape and perform volume rendering. This however requires computation intense deconvolution algorithms, which has been facilitated by improved software developments and ever increasing computing power.

In combination with genetic expression of markers we were able to determine the 3D shape of different groups of glial cells in the enteric nervous system of the mouse (Boesmans et al. 2014). Especially the structure of the type II glial cell is intriguing as it suggests that glial processes from one single cell are in close contact with most of the neuronal fibers that connect two ganglia in the myenteric plexus. Apart from its use in mouse tissue, the computational and reconstruction approach can also be applied to samples labeled with antibodies. One promising example of this quantification method applies to submucous ganglia in biopsies from the human intestine, as these have been shown to contain a small amount of neurons. On the one hand these neurons can be used to investigate marker expression with immunohistochemistry (Lebouvier et al. 2010) but also to record from while tissues are still alive (Cirillo et al. 2013). Volume reconstructions (Fig. 18.1) to determine abnormalities in neuronal processes or glial cell shapes may be useful to understand their association with diseases (Cirillo et al. 2015).

Apart from fixed tissues, also live tissues can be visualized using confocal technology. With one such approach, spinning disk confocal microscopy it is possible to record image stacks (~10 slices volume per second), which generates a 3D cube of information at a temporal resolution (1 Hz) comparable to what is available in 2D CCD camera based systems. The speed at which the recordings can be made

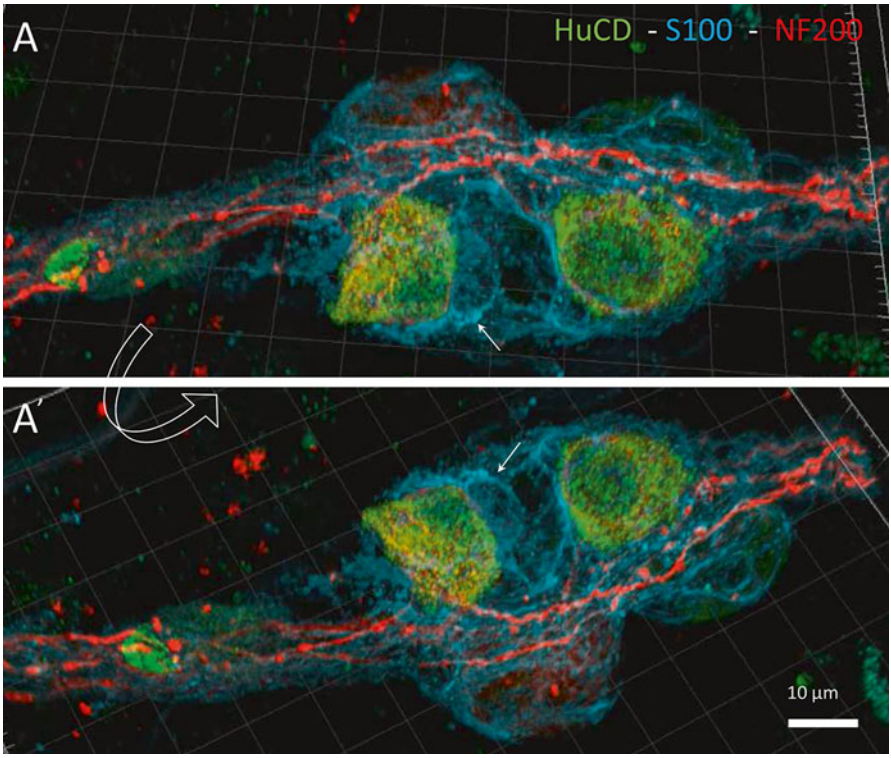

Fig. 18.1 Triple labeling of a human submucous ganglion present in a routine duodenal biopsy. (**A**) and (**A′**) are snapshots taken from a 3D confocal stack of images recorded on a Zeiss confocal microscope and deconvolved using Huygens (SVI). The *white arrow* indicates a glial cell (as identified by S100 antibody labeling) in close apposition to a nerve cell (HuCD) and neuronal fibers (NF200)

obviously depends on the hardware of the recording system. However this is often not the limiting factor, as recording speed may well be determined more importantly by the signal to noise (S/N) ratios that can be obtained. Using genetically encoded Ca^{2+} sensors like GCaMP (Zariwala et al. 2012; Boesmans et al. 2013) high signal to noise ratios can be obtained much more easily than with bulk dye loading techniques (e.g. Fluo4).

Fast Imaging

Virtually all knowledge about action potential generation and propagation is based on micro-electrode and patch-clamp techniques, which measure electrical signals directly. These methods have generated invaluable and crucial information about the nature of the underlying ionic conductances. Although electrical recordings

cannot be beaten in terms of temporal resolution, the spatial aspect of electrode recordings is very poor, as data can only be collected from a limited number of sites, mostly only one, being the postsynaptic cell.

Although voltage sensitive dyes (V_m dyes: di-4-ANEPPS, RH 484) have been around for several decades, their relatively poor quantum yield—compared to some Ca^{2+} indicators—has prevented widespread use of these dyes. Given the improved stability of later generation V_m dyes (di-8-ANEPPS) and enhanced sensitivity of the recording equipment (Obaid et al. 1999; Neunlist et al. 1999), V_m dye recordings have become a lot more efficient. In order to record these fast events dedicated cameras are necessary to collect a sufficiently large signal at kHz frequency. Maximum temporal resolution can only be obtained using widefield fluorescence microscopy in combination with high speed (EMCCD's and sCMOS) cameras designed for fast acquisition (e.g. Obaid et al. 1999, 2005; Neunlist et al. 1999; Buhner et al. 2009). In the enteric nervous system di-8-ANEPPS recordings have been used successfully to monitor action potential discharge and fast excitatory post synaptic potentials elicited by different mediators (e.g. histamine) but also by extracts from mucosal cells obtained from patients with inflammatory bowel diseases (Buhner et al. 2009).

As an alternative to voltage sensors, also Ca^{2+} indicators can be used to monitor neuronal activity. These bright fluorescent molecules have been used as a surrogate for recording nerve activity because of the tight correlation between action potential firing and cytosolic Ca^{2+} events, of which the amplitude is related to the number of action potentials (Hillsley et al. 2000; Vanden Berghe et al. 2002). Although Ca^{2+} imaging has provided invaluable information, with classic image recording speeds, Ca^{2+} transients appear as relatively uniform events. However, the initial rise in cytosolic Ca^{2+} rise involves many different processes such as Ca^{2+} channel opening, release and uptake from intracellular stores and mitochondria. We found that the upstroke in cytosolic Ca^{2+} proceeds stepwise (Michel et al. 2011), reflecting the contribution of individual channels or channel clusters. Ca^{2+} recordings with this level of detail are only possible with cameras that are fast (kHz frequency) and sensitive enough to collect the limited number of photons generated in a millisecond time period. In a recent paper we describe such a microscope configuration, in which speed is combined with relatively high resolution (512×512 pixel CMOS camera chip). This setup makes it possible to record fast Ca^{2+} transients in individual varicosities. This technique allows one to discriminate between pre- and postsynaptic activity in an all optical way (Martens et al. 2014) and is a promising tool to investigate synaptic circuits in the enteric nervous system.

Non-Linear Optical Approaches

When light hits matter it is either absorbed or scattered (reflection, refraction or diffraction), which renders objects color, make them transparent, reflective or opaque. These daily life optical interactions obey a linear law, in that the effect

produced bears a linear relationship with the intensity of the incident light. However this linearity does no longer hold true for high light intensities as generated by high power pulsed lasers. The ensemble of effects produced by high intensity light sources is commonly termed non-linear optics (NLO) and because NLO effects depend quadratically on the incident light intensity they have the advantage to only occur in a small focal volume. Probably the best known phenomenon is 2-photon (2P) absorption, which can be used to excite fluorescent molecules with photons double the wavelength than normally used for single photon excitation. This specific excitation method has a number of important advantages. First, excitation only occurs at a confined (confocal) spot, which reduces phototoxicity as molecules above or below focus are not excited. Not only is 2P excitation intrinsically confocal, infrared light also penetrates significantly deeper into tissues due to reduced scattering, which makes it possible to excite molecules deep into living tissues. Penetration depth is definitely not infinite but at the moment sufficient to image through the entire intestinal wall of small rodents (Fig. 18.2).

A second imaging technique that is more recent and also relies on a NLO effect is second harmonic (SH) imaging. Due to the high intensity of the incident light, frequency doubling can occur, whereby a small amount of light is generated with

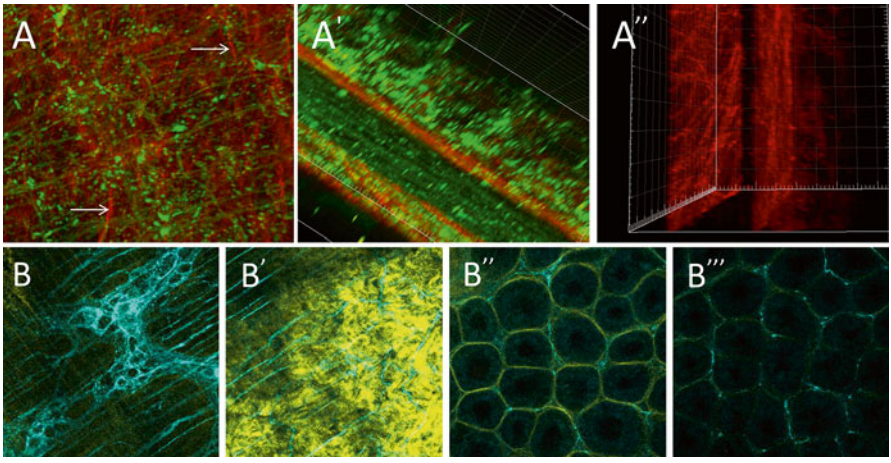

Fig. 18.2 Non-linear optical imaging of the mouse intestinal wall of the synaptopHluorin mouse (Li et al. 2005). This mouse expresses a green fluorescent protein in the fibers of some enteric neurons. Using 2-photon excitation (820 nm) it becomes possible to penetrate and image individual fibers as they extend into the mucosal layers in an intact and live intestinal preparation (*green* in **A**; *cyan* in **B**). Simultaneously with 2P excitation, frequency doubling occurs in the collagen layers (*red* in **A**; *yellow* in **B**). **A**, **A′**, **A″**: Shows snapshots of a 3D reconstruction of the synaptopHluorin as well as the collagen layers (*red*) in the mouse colon. Note that the collagen is present only in two very distinct layers (**A″**). (**B**): Confocal images taken at different depths in the synaptopHluorin mouse intestine. Fluorescence in *cyan*, second harmonic signals from collagen in *yellow*. Note that the fluorescence is readily excitable in myenteric and submucous plexus layers and remains detectable even several hundreds of μm into the crypt and mucosal layer of the intestinal wall (**B″** and **B‴**)

exactly double the frequency (half the wavelength) of the incident light. As this is intrinsically a scattering phenomenon, no absorption and therefore no photodestruction of a given molecule can occur. However not all molecules are capable of generating SH, only non-centrosymmetric molecules that are highly ordered display this effect. A number of endogenous biomolecules have this property including collagen, elastin and tubulin. Especially collagen generates SH very efficiently and can be used to image structural aspects of the intestinal wall (Fig. 18.2). Collagen and other extracellular matrix proteins, secreted by mesenchymal cells, cross link and arrange leading to altered physical fiber properties, which can be detected with these advanced optical techniques. We anticipate that this imaging technology will be useful to investigate structural changes that occur in inflammatory diseases like Crohn's disease (CD), which is characterized by relentless transmural inflammation of the intestine, leading to severe complications like fibrotic stenoses. Transmural strictures arise from extracellular matrix deposition (including collagen) and smooth muscle and myofibroblast hyperplasia. Several extracellular proteins secreted by mesenchymal cells (collagen 1-3, fibronectin) have been found in resection segments of patients with Crohn's strictures.

In conclusion, development of microscopy techniques is not at a standstill. Implementing novel microscopy strategies is of utmost importance to understand not only the cellular interactions in the planar ENS but even more so to investigate how information flows in three dimensions from the mucosa to the nerve layers, how this is influencing the control of blood flow and how that might depend on structural changes like collagen deposition.

Acknowledgements All members of the Lab for Enteric NeuroScience (LENS) are acknowledged for their conceptual contribution to the imaging approaches presented in this paper. Funding FWO-Vlaanderen (G.0501.10, G.0889.11, G.0929.15) and Hercules foundation (2009–2011).

References

Boesmans W, Martens MA, Weltens N, Hao MM, Tack J, Cirillo C, Vanden Berghe P (2013) Imaging neuron–glia interactions in the enteric nervous system. Front Cell Neurosci 7:183

Boesmans W, Lasrado R, Vanden Berghe P, Pachnis V (2014) Heterogeneity and phenotypic plasticity of glial cells in the mammalian enteric nervous system. Glia 63(2):229–241. doi:10.1002/glia.22746

Boesmans W, Hao MM, Vanden Berghe P (2015) Optical tools to investigate cellular activity in the intestinal wall. J Neurogastroenterol Motil 21(3):337–351

Buhner S, Li Q, Vignali S, Barbara G, De Giorgio R, Stanghellini V, Cremon C, Zeller F, Langer R, Daniel H, Michel K, Schemann M (2009) Activation of human enteric neurons by supernatants of colonic biopsy specimens from patients with irritable bowel syndrome. Gastroenterology 137(4):1425–1434

Cirillo C, Tack J, Vanden Berghe P (2013) Nerve activity recordings in routine human intestinal biopsies. Gut 62(2):227–235

Cirillo C, Bessissow T, Desmet AS, Vanheel H, Tack J, Vanden Berghe P (2015) Evidence for neuronal and structural changes in submucous ganglia of patients with functional dyspepsia. Am J Gastroenterol 110(8):1205–1215

Costa M, Brookes SJ, Steele PA, Gibbins I, Burcher E, Kandiah CJ (1996) Neurochemical classification of myenteric neurons in the guinea-pig ileum. Neuroscience 75(3):949–967
Furness JB (2000) Types of neurons in the enteric nervous system. J Auton Nerv Syst 81:87–96
Hillsley K, Kenyon JL, Smith TK (2000) Ryanodine-sensitive stores regulate the excitability of AH neurons in the myenteric plexus of guinea-pig ileum. J Neurophysiol 84(6):2777–2785
Hirst GD, McKirdy HC (1975) Synaptic potentials recorded from neurones of the submucous plexus of guinea-pig small intestine. J Physiol 249(2):369–385
Lebouvier T, Coron E, Chaumette T, Paillusson S, Bruley des Varannes S, Neunlist M, Derkinderen P (2010) Routine colonic biopsies as a new tool to study the enteric nervous system in living patients. Neurogastroenterol Motil 22(1):e11–e14. doi:10.1111/j.1365-2982.2009.01368.x
Li Z, Burrone J, Tyler WJ, Hartman KN, Albeanu DF, Murthy VN (2005) Synaptic vesicle recycling studied in transgenic mice expressing synaptopHluorin. Proc Natl Acad Sci U S A 102(17):6131–6136
Martens MA, Boesmans W, Vanden Berghe P (2014) Calcium imaging at kHz frame rates resolves millisecond timing in neuronal circuits and varicosities. Biomed Opt Express 5(8):2648–2661
Michel K, Michaelis M, Mazzuoli G, Mueller K, Vanden Berghe P, Schemann M (2011) Fast calcium and voltage-sensitive dye imaging in enteric neurones reveal calcium peaks associated with single action potential discharge. J Physiol 589:5941–5947
Neunlist M, Peters S, Schemann M (1999) Multisite optical recording of excitability in the enteric nervous system. Neurogastroenterol Motil 11:393–402
Obaid AL, Koyano T, Lindstrom J, Sakai T, Salzberg BM (1999) Spatiotemporal patterns of activity in an intact mammalian network with single-cell resolution: optical studies of nicotinic activity in an enteric plexus. J Neurosci 19(8):3073–3093
Obaid AL, Nelson ME, Lindstrom J, Salzberg BM (2005) Optical studies of nicotinic acetylcholine receptor subtypes in the guinea-pig enteric nervous system. J Exp Biol 208:2981–3001
Prasher DC (1995) Using GFP to see the light. Trends Genet 11(8):320–323
Vanden Berghe P, Kenyon JL, Smith TK (2002) Mitochondrial Ca2+ uptake regulates the excitability of myenteric neurons. J Neurosci 22:6962–6971
Wood JD (1989) Electrical and synaptic behavior of enteric neurons. In: Wood JD (ed) Handbook of physiology, the gastrointestinal system motility and circulation, section 6, vol 1. American Physiological Society, Bethesda, MD, pp 465–517
Zariwala HA, Borghuis BG, Hoogland TM, Madisen L, Tian L, De Zeeuw CI, Zeng H, Looger LL, Svoboda K, Chen TW (2012) A Cre-dependent GCaMP3 reporter mouse for neuronal imaging in vivo. J Neurosci 32:3131–3141