JNM
J Neurogastroenterol Motil, Vol. 21 No. 3 July, 2015
pISSN: 2093-0879 eISSN: 2093-0887
http://dx.doi.org/10.5056/jnm15096
Journal of Neurogastroenterology and Motility

Technique Review

Optical Tools to Investigate Cellular Activity in the Intestinal Wall

Werend Boesmans, Marlene M Hao, and Pieter Vanden Berghe*

Laboratory for Enteric NeuroScience (LENS), Translational Research Center for GastroIntestinal Disorders (TARGID), Department of Clinical and Experimental Medicine, University of Leuven, Leuven, Belgium

Live imaging has become an essential tool to investigate the coordinated activity and output of cellular networks. Within the last decade, 2 Nobel prizes have been awarded to recognize innovations in the field of imaging: one for the discovery, use, and optimization of the green fluorescent protein (2008) and the second for the development of super-resolved fluorescence microscopy (2014). New advances in both optogenetics and microscopy now enable researchers to record and manipulate activity from specific populations of cells with better contrast and resolution, at higher speeds, and deeper into live tissues. In this review, we will discuss some of the recent developments in microscope technology and in the synthesis of fluorescent probes, both synthetic and genetically encoded. We focus on how live imaging of cellular physiology has progressed our understanding of the control of gastrointestinal motility, and we discuss the hurdles to overcome in order to apply the novel tools in the field of neurogastroenterology and motility.

(J Neurogastroenterol Motil 2015;21:337-351)

Key Words
Calcium imaging; Enteric nervous system; Fluorescence; Gastrointestinal motility; Microscopy

Introduction

Live cell microscopy has become an essential technique for researchers who aim at monitoring cellular activity in integrated systems in order to understand the control of physiological processes. The most important advantage of live imaging is the fact that many cells can be monitored simultaneously, which, in contrast to electrical recordings that are mostly confined to single cells, allows the identification of cellular interactions and patterns. This is an extremely important asset in tissues where multiple cell types, sometimes sparsely located at critical locations, are working together to coordinate organ function. Moreover, because recording in 3 dimensions is possible and disruption of tissue integrity can be reduced to a minimum, live imaging aids in investigating genuine spatial relations and connections between cells.

One such organ where interactions between different cell types is crucial, is the gastrointestinal (GI) tract. Conveniently,

Received: June 2, 2015 Revised: None Accepted: June 10, 2015

*Correspondence: Pieter Vanden Berghe, PhD
Laboratory for Enteric NeuroScience, TARGID, University of Leuven, Herestraat 49, ON1, mailstop 701, Leuven B-3000, Belgium
Tel: +32-16-33-0153, Fax: +32-16-34-5939, E-mail: pieter.vandenberghe@med.kuleuven.be
Financial support: This study was supported by University of Leuven (BOF, OT 0501.10), the Fonds voor wetenschappelijk onderzoek (FWO, G.0501.10 & G.0921.15), NHMRC, and Hercules Foundation Flanders.
Conflicts of interest: None.
Author contributions: Werend Boesmans, Marlene M Hao, and Pieter Vanden Berghe researched literature and wrote the manuscript.
ORCID: Werend Boesmans, http://orcid.org/0000-0002-2426-0451, Marlene M Hao, http://orcid.org/0000-0002-9701-8252, Pieter Vanden Berghe, http://orcid.org/0000-0002-0009-2094.

the gut is organized in well-defined spatially restricted layers: 2 nerve plexuses (myenteric and submucous) with many other cell types embedded within muscle layers and connective tissue,[1] assisted by interstitial cells,[2] different types of glial cells,[3,4] and seeded at crucial locations with immune cells,[5] all of which interact in one way or another with vasculature, epithelial and enteroendocrine cells in the mucosal epithelium. Although intriguingly complex, the concentric tissue sheets that shape most of the digestive tract make the gut ideal to be studied by live imaging techniques. In particular, the enteric nervous system (ENS) and associated cellular systems responsible for the independent control of GI functions such as intestinal motility, can be filmed to understand gastrointestinal physiology in an integrated ex vivo organ setting. Remarkably, although Bayliss and Starling's description of "the law of the intestine: already dates back more than a century,[6] their discovery is still puzzling many researchers in the field of neurogastroenterology and motility today. Major breakthroughs that advance our comprehension of the development and function of the cellular components intrinsic to the gut wall have always relied on a strong microscopy component, but compared to the progress made in, for example, central nervous system (CNS) research, the neurogastroenterology field is lagging behind in developing and applying highly innovative imaging techniques. A striking example is the recent "optogenetics revolution" which provides tools to manipulate and record activity in cellular systems with the spatial, temporal, and cell-type resolution that is governed by optical interaction with genetically encoded proteins.[7] This has led to an explosive growth in brain research strategies but so far, has not got a convincing foothold in the ENS community.

Imaging technology obviously also has its limitations, as high quality imaging critically depends on the availability of efficient probes that can translate physiological processes (for example, changes in specific ion concentrations, an action potential, a contraction, a membrane fusion, a release event, etc) into photons. Secondly, there is a limit to the optical resolution that can be obtained because light, as any electromagnetic wave, is subjected to diffraction as it propagates through optical components and diffractive media. Lastly, there is also a limit to the current recordings speeds of imaging systems, which becomes an issue for the faster electrophysiological events, especially in neurons. However, in the last decade, some important progress has been made to tackle the limitations of live imaging and microscopy, not only in terms of technical developments but especially with respect to the spatial and temporal specificity of delivery methods for probes and reporter molecules.

Although the technology itself is critically important, in this review, rather than expanding on microscopy techniques, we chose to mainly address the different fluorescent probes that are now at hand to study and manipulate cellular and tissue activity. In the first section, we focus on a number of important scientific achievements that have shaped the recent advances in tools and subsisting needs for live imaging microscopy. We then give an overview of the synthetic dyes and their genetic "homologues," each with their advantages and disadvantages, and we dedicate a chapter to the novel optical tools to manipulate cellular function. In the last section, we will discuss what their use has recently taught us about cellular function and interactions in the intestinal wall, and conclude with a number of future perspectives.

Live Imaging Microscopy: Moving Forward

Probes

One crucial aspect of microscopic imaging is the need to generate contrast between the item of interest and its background. In fluorescence imaging, this contrast can be generated by adding fluorescent molecules in the cells of interest. Originally, molecules such as Lucifer yellow were used to understand how clusters of cells were coupled to each other, by studying the spread of dye from cell to cell.[8] Later on, dyes were developed where the fluorescent properties depend on a physiological state (eg, membrane potential) or on an intracellular ion concentration (eg, H^+, Ca^{2+}, K^+, and Na^+). These sensors have been very useful to monitor physiology as they immediately translate the cellular event into numbers of photons and therefore signal intensity. Instead of being ubiquitously located throughout the cytosol of cells, these labels have also been further developed to generate dyes that partition into specific organelles (mitotracker and lysotracker), or label actively recycling membrane parts (FM1-43), or incorporate into DNA (DAPI and Hoechst). Although these small molecules have proven their efficacy, they share one problem where the bulk loading procedure that is generally used to introduce them into cells does not really discriminate between different cell types.

The advent of genetically encoded fluorescent molecules has caused a huge advance in the field of live cell imaging. Most of the genetically encoded probes are based on the green fluorescent protein (GFP), which had been isolated from the jellyfish

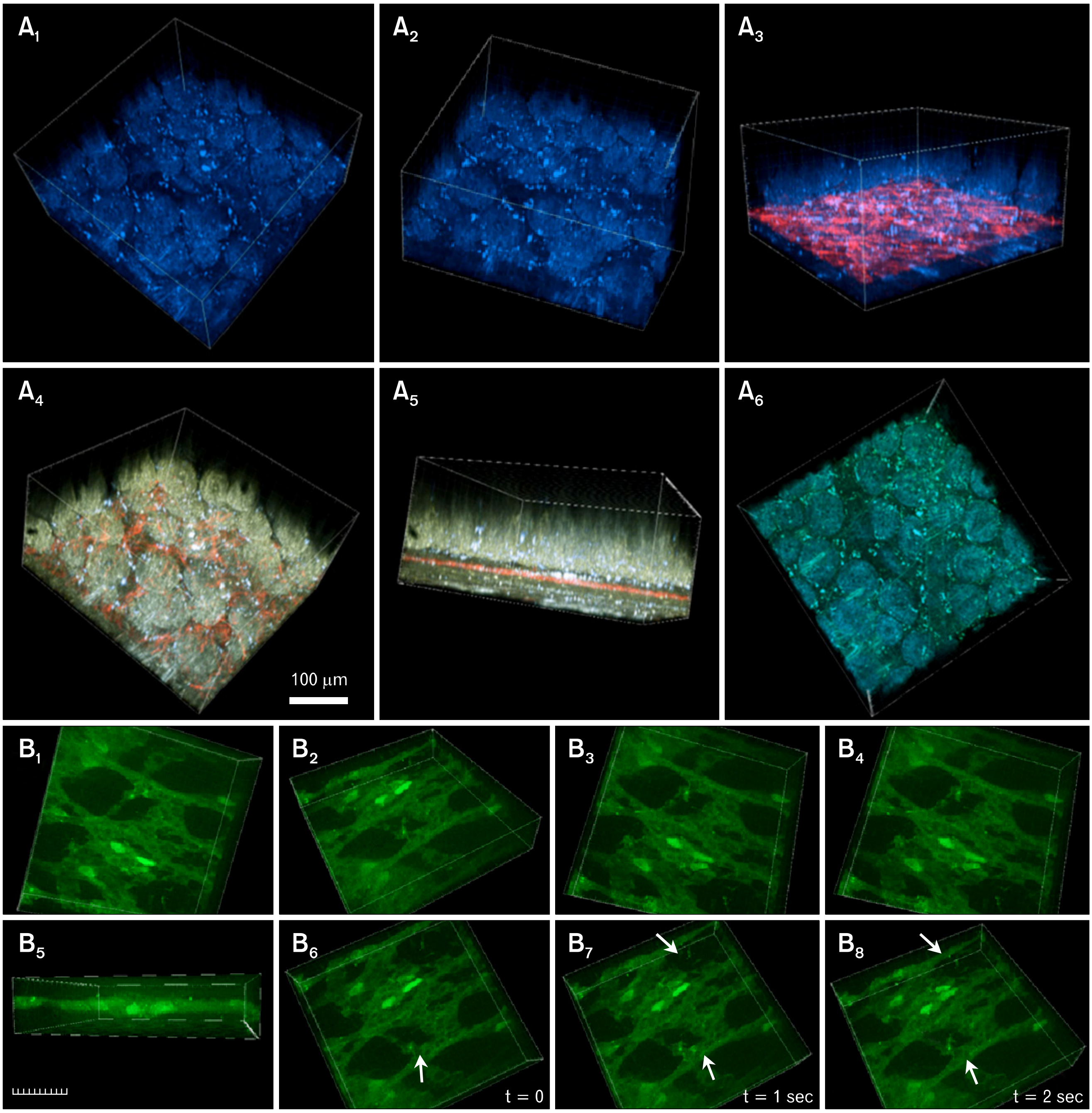

Figure 1. Three-dimensional (3D) optical recordings of living tissue of the mouse intestinal wall. (A) Six sequential projections of a 3D rendering computed from a 2-photon fluorescence recording of live adult mouse intestine (A_{1-6}). To enhance contrast, DAPI was applied onto the luminal side, as shown in blue and yellow. No other labeling is present. The autofluorescence of endogenous molecules is shown in green; the green speckles apparent in A_6 are chlorophyll containing remnants of chow. In red is the second harmonic signal of the collagen layer. (B) Snapshots of a 3D live recording of the intestinal wall of a P0 *Wnt1-Cre;R26R-GCaMP3* mouse jejunum. B_{1-5} show different projections that allow the 3D appreciation of myenteric ganglia, B_{6-8} show 3 timepoints during which a myenteric neuron shows spontaneous Ca^{2+} transients that can be clearly followed in an upward projecting process (arrows).

Aequorea victoria.[9] In 2008, Dr. Tsien, Dr. Shimomura, and Dr. Chalfie were awarded the Nobel Prize in Chemistry for their work on GFP, which has revolutionized the way biological structures and processes are visualized. In addition to GFP, coral proteins have also been engineered to yield functional, mostly red-shifted reporter proteins. This has furthermore led to a rapid progress in the discovery and generation of genetically encoded reporters, sensors and actuators. Protein sensors have the advantage that a biological interaction with another protein, a second messenger (Ca^{2+} and cAMP)[10,11] or a targeting sequence can be exploited to generate specificity of the sensor to a particular target or be directed to a distinct location within the cell. Combined with the currently available transgenic animal models, viral vector technology and improved gene transfer methods, it is now possible to label, monitor and manipulate specific cell populations using optical tools. This is an invaluable asset as it circumvents the major limitation shared by many of the small molecule dyes used in neuroscience and physiology, which is that their universal nature generally hinders targeting specific cell types.

Microscopy Techniques

Need for contrast and resolution

Although for most cellular imaging, optical resolution has not been a realistic hurdle, it is important to consider the limitations of optical microscopy, especially when conclusions are drawn about signals arising from subcellular or overlapping structures. To overcome this problem, super resolution approaches have been developed over the last 20 years.[12-15] Three of the crucial researchers in this field were awarded the Nobel Prize in 2014 for their contribution towards circumventing the diffraction barrier and improving optical resolution.[16] Although tremendous improvements have been made, this newly developed technology is still not straightforward for use in live cell imaging. In the case of stimulated emission depletion (STED), the optical powers required are still very high (many orders of magnitude higher than necessary as in, for example, confocal microscopy), which jeopardizes its use for most, but not all[17] live imaging approaches. As for the stochastic superresolution techniques (photoactivated localization microscopy [PALM] and stochastic optical reconstruction microscopy [STORM]), in which the extra resolution is based on the fact that only a limited number of molecules is illuminated and recorded from at same time, total image reconstruction can take up to minutes and is therefore not yet suited for recording fast cellular events.

Offline image analysis techniques (deconvolution and spatio-temporal correlations) can also be applied, but far-stretching conclusions about cellular interactions, receptor presence, etc. should be avoided when studying tissues stained with bulk loading procedures. There are solutions to the problem of resolution that do not require very expensive experimental equipment or advanced image analysis, but rather the appropriate location or expression of fluorescent probes themselves can generate sufficient contrast. This can be achieved by local application or injection of dyes to confined structures or by genetic targeting of fluorescent reporters, especially when sparse labeling is induced. In addition, one can also combine a genetic labeling technique in combination with the application of a synthetic dye. By restricting the read-out of the functional probe to the region or preferably volume of interest, it is possible to make a reliable judgement of how specific cells respond to a given stimulus.

Need for speed

Another important limitation of imaging, especially with respect to monitoring neuronal activity, is that acquisition speeds are far worse than those of electrical recordings, which can easily generate thousands of datapoints per second. Continuous improvements of lens quality, camera resolution and sensitivity have partially helped to overcome this limitation, in that more photons can be collected in short (millisecond) timespans. Cameras that operate at kilohertz speeds have been used successfully[18-21] but their price, unfortunately still puts a brake on the use of voltage sensing molecules, for which kiloHertz acquisition rates are a prerequisite.

Need for depth

In order to preserve and record from integrated 3-dimensional (3D) cellular networks in situ, imaging strategies are necessary to penetrate deep into the tissue. Confocal and especially multiphoton microscopy are excellent tools to optically select layers within living tissues, but tissue scattering remains the most important determinant for imaging depth. Although longer wavelengths, as used in multiphoton imaging penetrate better into tissue, visualizing structures deeper than 1 mm is not realistic. For brain tissue that means imaging is restricted to the cortical layers (I to V), but fortuitously, this imaging depth is sufficient to reach through the intestinal wall (Fig. 1A). Here again, (genetic) labeling of specific cell layers brings extra contrast and helps to, in combination with confocal or multiphoton technology, generate high quality images from deeper layers (Fig. 1B and supplementary Movie). Obviously not suitable for live imaging approaches, but for fixed tissues a number of clearing techniques have been developed to suppress refractive index variations. Clarity[22] and iDISCO[23] are 2 such techniques that are mainly applied to entire brains and embryos respectively. In the GI field impressive 3D recordings in cleared intestinal tissue have also been reported.[24,25]

How to Sense Physiological Events

The earliest reports on imaging of cellular activity date back about 40 years[26,27] when researchers first started using voltage sensitive dyes to report changes in membrane potential in the squid giant axon. Soon after, it was realized that ion sensors could in many ways replace voltage sensors, which were, and still are, much more tedious to use. In particular, Ca^{2+} sensors turned out to be a useful alternative, as intracellular Ca^{2+} is a fairly ubiquitous second messenger and its concentration ($[Ca^{2+}]_i$) changes upon neuronal action potential firing. Ca^{2+} signaling is also somewhat more general as it can be used to monitor receptor mediated responses that do not necessarily generate large membrane depolarisations. Last, because the Ca^{2+} signals become amplified by Ca^{2+} stored in the endoplasmic reticulum, the change in reporter fluorescence is likewise enlarged and easier to record. With respect to neuronal signaling, the most important drawback remains that Ca^{2+} signals only—though very fiducially[19,28,29]—reflect the consequence of electrical activity that has occurred in a cell. Although not outweighing the advantages of the excellent signal to noise ratios of Ca^{2+} reporters, it is important to remember that this link to membrane potential changes is indirect. In the same context it is important that terminology such as "action potential firing," or "depolarization" should be avoided when using $[Ca^{2+}]_i$ signals.

This issue becomes even more challenging when non-excitable cells are also subjected to Ca^{2+} imaging. It remains unclear as to what exactly happens in glial, epithelial or immune cells when a rise in $[Ca^{2+}]_i$ is observed. Nevertheless it remains an important and useful readout as it indicates that the studied cell or tissue has been perturbed from its normal equilibrium, indicating at least some sort of "activation." With the basics of intracellular Ca^{2+} household[30] in mind, one can carefully draw the correct conclusions for each and every cell type. The complexity of ion imaging, as already recognized a little over 20 years ago,[31] should not be underestimated and correct use and interpretation of the technique need to be performed with care.

Interestingly, for almost all synthetic fluorescent probes that have been developed to monitor cellular activity, a genetically encoded counterpart has also been generated. This parallel indicates that the importance of optical imaging has not waned, but on the contrary, has been growing and incorporating new technology. It is important to mention that the genetically encoded counterparts do not bring full relief, as the synthetic dyes are still superior in terms of their reaction speed (ie, speed in change of fluorescence intensity) and their molecular size is generally much smaller.

Voltage Indicators

The first generation of synthetic voltage sensors were developed in the early nineties and in particular, di-8-ANEPPS has been shown to be the dye of choice for monitoring membrane potentials.[32] This dye has a sufficiently large change in its fluorescence ratio per millivolt change in membrane potential (2-10%/100 mV) and has a fast on and off rate.[21] Recently some other voltage sensitive dyes have been reported: JPW-1114[33] and FLIPR membrane potential dye,[34] but neither have obvious superiority for general use as the first needs to be loaded intracellularly and the latter has kinetic constants in the order of 4-8 seconds, which is significantly slower than di-8-ANNEPS and is not linearly dependent on the membrane potential. The FLIPR membrane potential dye however has an advantage over the other voltage sensitive dyes in that it displays an increase in fluorescence for a positive deflection of the membrane potential. A drop in fluorescence for increases in membrane potential, which is common to most voltage sensors, generally means that the dyes are more prone to photobleaching in their resting state.

Apart from synthetic dyes, genetically encoded voltage indicators (GEVI) have been designed to make targeting of specific cells possible.[35,36] The voltage sensitivity of many of the GEVIs (voltage sensitive phosphatase) is based on conformational changes in the protein which either directly, or indirectly based on Förster Resonance Energy Transfer (FRET), alter the fluorescent properties of the reporter. However, the conformational change limits the temporal resolution, making recordings faster than 200 Hz not realistic. The use of GEVIs seems to be concentrated in the field of (live) cortical imaging, where often mesoscopic compound signals are recorded,[37] for which cellular resolution and resolving individual action potentials is not a requirement. Newer generations of GEVIs have improved speed as they utilize the intrinsic voltage sensitivity of rhodopsins (Arch[38]) or mutated GFP's (ArchLight[39]). Mutations in the Arch protein generated yet another GEVI (QuasAr[40,41]) with greater sensitivity, higher signal-to-noise ratios and better temporal resolution.

Ion Sensors

Although voltage sensors are obvious tools for imaging action potential firing, the ease of use, the larger positive changes in fluorescence and the greater stability of ion sensors, in particular those for Ca^{2+}, has surpassed the use of both synthetic and genetically encoded voltage sensors.[36] In terms of small synthetic molecules, the developments have stagnated somewhat, the usual suspects Fluo-4 and Fura-2 are probably the best known and most used. Fluo-4 is used more commonly in tissues and when measuring relative changes in Ca^{2+} suffices, while Fura-2 is needed when actual Ca^{2+} concentrations need to be computed. Several other synthetic dyes that can be used for bulk loading of tissues exist and all have their different spectral properties and sensitivities.[42]

Similar to voltage sensors, the first generations of genetically encoded Ca^{2+} indicators (GECIs) were based on large protein conformation shifts, that allowed FRET signals to be recorded.[43] An impressive improvement was made when instead of the FRET principle a circularly permutated GFP was fused to calmodulin and M13, a synthetic peptide from myosin light chain kinase. Upon binding to Ca^{2+}, these proteins slightly deform the protein barrel of GFP to increase its fluorescence intensity.[10] After rounds of mutations, GCaMP3 was designed, which has now been used in many different neuronal systems.[44-46] However, its detection reliability of single action potentials is still relatively low under physiological conditions.[47] The availability of a transgenic mouse line in which GCaMP3 is expressed conditionally (by Cre-Lox technology) from the *Rosa26* locus[48] has made GECI imaging very accessible. Random mutagenesis has generated a series of GCaMP based mutants with different spectral properties, that were termed G (green), R (red) GECO's (Genetically encoded Ca^{2+} indicators for Optical imaging), however none of them with obvious improvements in quality.[49] However, specific modifications of the GCaMP construct has yielded improved versions either with respect to the amplitude of fluorescence change (GCaMP5[50]) or speed (GCaMP6[51] and GCaMP8[52]). Even though the newer GCaMPs have improved characteristics, the protein conformation change that is required for all GCaMP molecules will always limit the speed at which these sensors operate. Thus, as with voltage sensors, the GECIs generally do not beat the synthetic dyes in terms of speed, but are superior when it comes to selective expression in certain cell types. Red shifted variants have also been developed and compared in terms of spectral and kinetic properties with other GECIs.[53] Here again, these proteins are not yet comparable with respect to the signal to noise properties and the ease of use of GCaMP3. For a comprehensive overview of the various probes and techniques to perform intracellular Ca^{2+} imaging, we refer to an excellent recent neuron-focused review by Grienberger and Konnerth.[54]

The use of sensors for other ions (K^+ and Na^+) has been very limited so far, as they are either not very sensitive, the concentration changes of these ions are never that explicit or they do not show the same ion selectivity as the Ca^{2+} sensors display. One exception is the H^+ sensors of which the genetically encoded pHluorins have proven useful as tags to monitor intracellular or intra-organelle pH differences, and as such can be used to monitor synaptic vesicle recycling.[55,56] Again, genetic engineering has led to improved versions based on fusion proteins with synaptophysin (SyPhy[57]), or red shifted variants.[58] The synthetic counterpart of these synapto-pHluorins are the styryl dyes such as FM1-43[59] that partition in the membrane and are co-recycled when membranes are retrieved from the presynaptic terminal.

Another group of labels worth mentioning are the organelle labels, which can be used to monitor trafficking events.[60-62] Here as well synthetic dyes (mitotracker and lysotracker) have been complemented by genetic approaches, which rely on the specificity of targeting sequences (eg, mito: *cox8* targeting sequence) tagged to the fluorescent protein of interest. This approach allowed Misgeld et al[63] to generate transgenic mice that have cyan fluorescent protein specifically in their neuronal mitochondria.

Although the advantages of genetic expression possibilities are numerous, there are also some limitations to consider when fluorescent proteins are used as reporters of cellular activity. First of all, compared to synthetic labels, the protein reporters are quite large and fusion proteins with a GFP based label carry at least that ~27 kD[9] extra weight. Second, most fluorescent proteins display blinking,[13] which is an important property that may interfere with single molecule detection or very fast recordings. Third, most of these protein reporters are slower in their responses, as protein conformational changes are inherently slower than pure electronic shifts in the fluorochromes. Also, specifically for GECIs, the dissociation constant (K_d) that determines their sensitivity to Ca^{2+}, is generally higher than for synthetic Ca^{2+} dyes and varies much more depending on pH or the specific location within the cell. Moreover, it is not clear how much the long term presence (depending on the method used to induce GECI expression) as opposed to the acute introduction (in case of synthetic dyes) of an extra Ca^{2+} buffer impacts on the physiology of a particular cell type. Lastly, another apparently counterintuitive but practical disadvantage, is the fact that a lot of mutated variants with improved characteristics are made available at a rate that greatly exceeds the speed of use and testing. This applies to a number of different GECIs, GEVIs, and opsins (see below). However, none of these disadvantages should in no way prevent or delay the use of these tools, but are important to take into account when detailed quantification is intended.

Optogenetic Modulation of Cellular Activity

Apart from the development of sensors or reporters that translate a cellular event into photons, also the reverse has been put into action in an impressive way.[64,65] The fact that light can interact with living matter is known to everyone; it can be used to confer heat (red shifted lamps) or when used at high powers or at certain wavelengths (UV) can perturb a cell's equilibrium or destroy biomolecules. However, the idea to use engineered proteins to accurately control cellular activity by light in a cell specific and selective manner dates only little over a decade. The earliest attempts took advantage of the protein machinery available in the Drosophila eye, from which 3 proteins were shown effective at activating mammalian cells (chARGe).[66] The search for a method to avoid co-expression of several proteins, led Miesenböck to genetically modify TRP and $P2X_2$ channels, to immediately couple light sensitivity to ion channel opening.[67] However, in the meantime the intrinsic light sensitivity of channelrhodopsin (ChR1 and ChR2) had been reported, a protein isolated from the alga *Chlamydomonas reinhardtii*, which displayed large photocurrents upon illumination (Fig. 2).[68] These could be used to control *Caenorhabditis elegans* behavior, which was shown 2 years later.[69] In the same year, Boyden et al[70] also reported that indeed ChR2 can be used to elicit realistic action potential trains in neurons. Channelrhodopsins have been since mutated in order to display better temporal characteristics (ChETA[71] and Ch(i)EF,[72]), become switchable to on and off states (SFO[73]) or carry larger

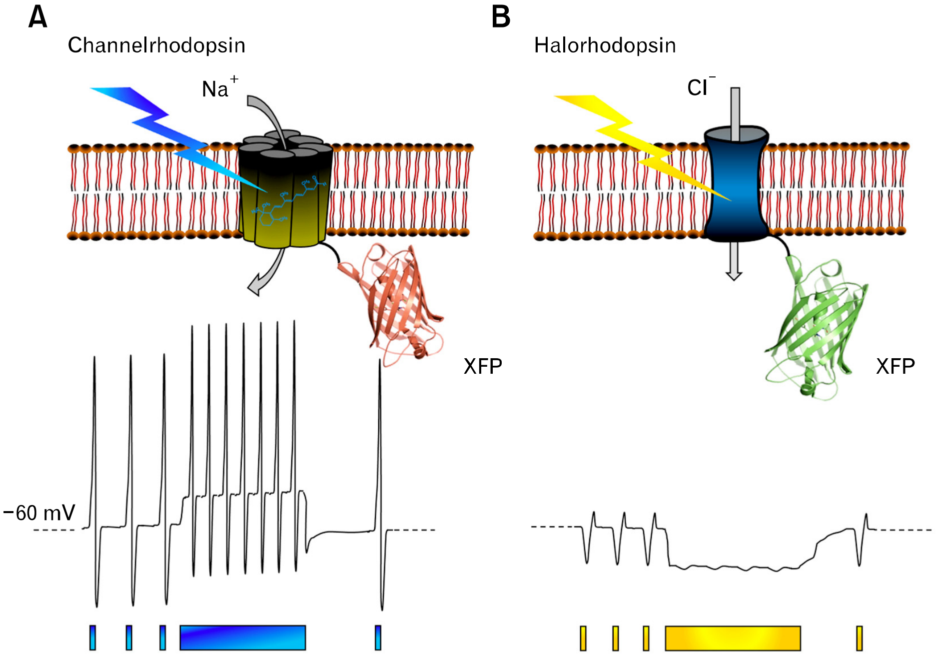

Figure 2. Schematic representation of the 2 most important opsin families. Upon blue illumination, Channelrhodopsins (derived from *Chlamydomonas reinhardtii*) will conduct cations, which in neurons, will result in Na^+ influx and a depolarization of the cell. A single action potential or more sustained depolarization can be elicited using either brief or longer light pulses. Halorhodopsins (*Natromonas pharaonii*) cause the opposite effect, in that upon illumination with yellow/orange light a Cl^- pump is switched on, leading to hyperpolarization of the cell. In order to know where the transgene is expressed, the opsins are generally fused to a fluorescent reporter protein (XFP), which should be carefully selected, as the wavelength to activate the opsins should not interfere with the wavelength used for visualization of the cell.

photocurrents.[74] Additionally, red-shifted channelrhodopsins derived from *Volvox carteri* (VChR1) have been developed.[75] Inhibitory opsins have also been developed, the best known being the (enhanced) halorhodopsins (eNpHR) derived from *Natromonas pharaoni*, which pumps Cl^- ions upon illumination and therefore hyperpolarizes neurons (Fig. 2).[76] Often, the optogenetic expression cassettes also include a fluorescent reporter tag that aids in the localization of the cells expressing these actuators. As with any genetically encoded system, these actuators can be expressed site and cell specifically or can be used to examine localized events such as synaptic function.[77]

Technically, microscope techniques are not needed for optogenetic actuators such as channelrhodopsins and halorhodopsins, as light can be delivered via optic fibers into specific locations of the brain or other organs. As such, optogenetic technology has been used in the study of many different diseases (eg, autism[78] and Parkinson's disease[79,80]) and behavioral experiments (eg, respiration,[81] locomotion,[82] and fear[83]).

At present an extensive, and at times bewildering,[84] palette of optogenetic tools is available. However, no matter how complete the toolkit has become, the choice of actuator and auxiliary driver suited for a specific experiment, together with the powers and wavelengths of light needed to discriminate between identification and modulation of the actuator-expressing cells, requires careful consideration by the researcher.

Applications of Live Fluorescent Imaging Techniques in Neurogastroenterology

Live Imaging of the Developing Enteric Nervous System

The ENS is a vital component in the control of GI function. All neurons and glia of the ENS arise from neural crest cells that migrate into the developing gut during development. These enteric neural crest-derived cells (ENCCs) proliferate, differentiate, and project neurites to appropriate target cells.[85-87] Live imaging has contributed to our understanding of 2 important aspects of ENS development: (1) the migration of ENCCs in the gut and (2) the development of neural activity in the immature ENS.

Live time-lapse imaging using different genetically-encoded fluorescent reporters has been crucial to investigate the migration of neurons and precursors within many parts of the developing nervous system, as well as various populations of neural crest cells.[88-90] In the ENS, live imaging using *Wnt1-cre;R26R-YFP* and $Ret^{TGM/+}$ mice, where yellow or green fluorescent proteins are expressed by neural crest-derived cells has demonstrated that ENCCs have a particular mode of migration in the gut, as cells remain mostly in contact with each other in "chains."[91-93] To enhance the cellular resolution within the migrating population, different genetically-encoded photo-convertible fluorescent proteins are now available. Photostimulation of these proteins, usually using violet to blue wavelength light, induces a change in the fluorescent properties.[94] There are 3 main types of photo-transformable fluorescent proteins: (1) photo-activatable proteins, where fluorescent emission is induced after stimulation (eg, photo-activatable GFP); (2) photo-convertible proteins, where stimulation produces a shift in the fluorescent emission spectra (eg, Kaede,[95] KikGR,[96] and Dendra,[97] which change from green to red emission); and (3) reversibly switchable fluorescent proteins, where the change in colour can be reversed (eg, Dronpa).[98] In the GI tract, *Ednrb-kikGR* mice, in which the photo-convertible protein kikGR is expressed in the ENS, have been used to examine the migratory behaviour of ENCCs.[99] Using this mouse, photo-conversion of single or small populations of cells allows the tracking of individual red cells within an otherwise homogenously labeled green population. This has resulted in a detailed description of the speed and direction of migration of individual cells[100] as well as the identification of a new pathway of migration, where ENCCs in the midgut "skip" across the mesentery to colonize the colon.[99] These "trans-mesenteric" migratory cells make up the majority of the ENS in the colon, and are therefore vital for complete colonisation of the gut.

To examine the development of ENS circuitry, activity of enteric neurons has been imaged using Fluo-4[101] and *Wnt1-Cre;R26R-GCaMP3* mice.[102] Initially, Fluo-4 Ca^{2+} imaging was performed on ENCCs isolated from different embryonic ages to examine $[Ca^{2+}]_i$ responses to electrical field stimulation.[101] More recently, the availability of a conditional GCaMP3-expressing mouse line has allowed Ca^{2+} imaging to be performed on intact explants of embryonic gut, thereby preserving the native cell-cell connections of the developing ENS.[102] Using the *Wnt1-Cre* transgene to induce expression of GCaMP3 in all neural crest derivatives, the contribution of different subunits of nicotinic receptors to cholinergic neurotransmission throughout ENS development has been characterized.

Live Imaging of Cellular Activity in the Adult Gut

The physiology of the cellular apparatus involved in the independent control of GI function has been studied in many classical electrophysiology experiments. Combined live imaging and electrophysiology of enteric neurons have confirmed that changes in membrane potential can be monitored both by Ca^{2+} and voltage-sensitive imaging,[19,28,29] although as described above, fluctuations in $[Ca^{2+}]_i$ can be determined by factors other than the membrane potential. Ca^{2+} and voltage-sensitive dyes have since allowed visualisation of activity of various cell types embedded in the GI wall on a larger scale. However, as opposed to many live imaging studies focusing on other parts of the nervous system, only few reports have examined activity patterns of neuronal circuits underlying integrated ENS output such as for example the colonic migrating motor complex.[103-105] Nevertheless, imaging of cultured enteric neurons and ex vivo tissue preparations have clarified important physiological characteristics of enteric neurons such as their mechanosensitivity, and thereby challenged the classic ideas on sensory transmission and reflex activity in the ENS.[106,107] The majority of studies in the gut have used classic live imaging techniques and synthetic indicator dyes, to monitor activity of the various cell types that govern GI motility. Although significant differences exist depending on the type and location (layer) of the cells of interest, bulk loading of dissected tissue has proven sufficiently useful.

Ca^{2+} imaging studies have also progressed our understanding of other than the intrinsic neuronal elements involved in the control of motility. It has been shown that enteric glial cells tune in to neuronal activity[108,109] and take part in ENS signaling that underlies colonic motility.[110,111] Also, our understanding of myogenic and other mesenchymal control elements present in the gut wall, has improved based on imaging results. For example, recent reports show that interstitial cells of Cajal can operate independently from enteric neurons to control segmentation motor activity,[112] and need the Ca^{2+}-activated Cl^- channel Ano1 to coordinate slow waves in the smooth muscle.[113] Another recent study used Oregon Green BAPTA-2 as a Ca^{2+} indicator to investigate the involvement of platelet derived growth factor receptor α ($PDGFR\alpha^+$) cells in inhibitory neurostransmission to smooth muscle cells.[114] The extrinsic innervation to the gut has also been examined in a recent Ca^{2+} imaging study,[115] in which activation of spinal afferents was detected upon mechanical distension of the colon. Furthermore, live imaging has also been used to address the interaction of the ENS with the immune system, and recent reports focused on the cholinergic modulation of resident macrophages.[116,117] A review on where to go with imaging of mast cell-nerve interactions has been published by Schemann and Camilleri.[118] Apart from using animal tissues, live recording from human ENS has also been achieved as samples from human patients are more accessible in comparison to most other nerve tissues. Voltage and Ca^{2+} recordings of enteric neuronal activity have been performed on tissue samples taken from human volunteers during surgery.[119,120] or with standard biopsy forceps.[121]

In addition to activity at the level of cell bodies, information about the transport and activity of organelles and subcellular structures is also instrumental in understanding enteric neural circuit function. As such, synaptic vesicle recycling has been monitored using the FM1-43 dye[122,123] and mice expressing synaptopHluorine.[124] Live imaging of mitochondrial transport along enteric neuron processes has so far been restricted to in vitro studies.[62,125]

Gastrointestinal Specific Imaging Problems

Despite the advantages conferred by live microscopy, there are still many difficulties to overcome in order to examine the enteric neural circuitry in its entirety and identify the specific contribution each cell type to control of GI motility. Researchers in the field of neurogastroenterology have to face the intriguing but specific setting of the GI tract. The ENS is situated in the highly heterogeneous cell environment of the gut wall and is layered in close apposition to contractile sheets of smooth muscle syncytia, thereby complicating several experimental approaches to a large extent. Especially in the context of live microscopic imaging, it is exactly the output of the enteric nerve circuits (ie, motility patterns) that hampers their detailed analysis. Although movement artifacts can be corrected using offline stabilization routines,[108,126] accurate analysis of small structures and cellular compartments remains challenging.

There is currently a limit in the ability to introduce either synthetic or genetically-encoded indicators into multiple types of cells in the gut tissue whilst preserving its 3D structure and tissue integrity. For instance, bulk loading of synthetic dyes, which has classically been used in many experiments, has some disadvantages. First, it requires the removal of many layers of tissue in order to penetrate to the cells of interest. Second, the dye usually enters different cell types indiscriminately, which may be advantageous, as many different cells can be imaged simultaneously. However, the majority of dyes do not enter all cells equally, thereby, distorting the output. For example, the lack of response in a particular cell type may be due to the fact that they are not well-loaded, and not necessarily because they do not respond to the applied stimulus. To avoid this, local application of synthetic dyes, or intracellular injection can be used, however, in this case, only specific cells can be examined. In addition, it is difficult to avoid some peeling of the gut mucosa as it is notorious for its autofluorescence, which can decrease the signal-to-noise ratio, especially when using green fluorescent dyes. Genetically encoded optical probes have begun to be used in ENS research, in particular GCaMP3.[102,108] However, so far the application of genetically-encoded probes has been restricted to those available in transgenic reporter mice. As described above, novel and improved versions of genetically-encoded sensors and actuators are constantly added to the already impressive list, but alternative methods for introducing novel genetic constructs into enteric neurons and other cells of the GI tract in vivo have not been reported. One explanation may be that the location of the ENS, close to the hostile and microorganism-crowded gut lumen, has developed increased resistance to the easy introduction of foreign genes via transfection or transduction protocols to prevent unwarranted DNA exchange. Unfortunately, this has restricted the ENS field in using the plethora of expression constructs that are being newly developed at an extraordinary pace. The successful transduction of enteric neurons with adeno-associated viral vectors that has been reported by a few new studies could be a

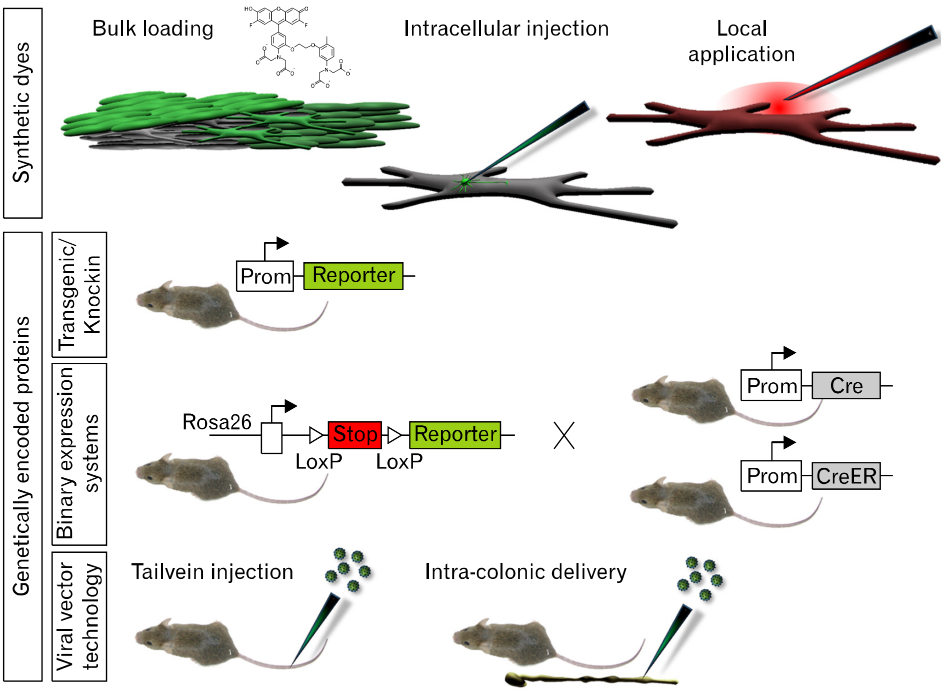

Figure 3. Schematic overview of possible strategies to deliver optical probes (synthetic and genetic) into intestinal tissues. The top row shows 3 methods to apply small synthetic dyes to ganglia, interstitial cells, and muscle layers. During bulk loading tissues are incubated in a buffer containing an AM-ester of a Ca^{2+} indicator (common examples are: Indo-1,[133] Fluo-3,[134] and Fluo-4[135-137]), Oregon Green BAPTA,[114] Rhod-2,[3] etc). The esters are cleaved by intracellular esterases, whereby the indicator becomes functional and is trapped within the cell. With bulk loading, the outermost layers will have higher levels of dye than the inside layers. Using sharp (or patch) electrodes Ca^{2+} indicators can also be loaded in individual cells,[28,29] or alternatively, dyes can be applied locally as often done with di-8-ANEPPS[20] to reduce labeling of other layers in the field of view. Strategies to express genetically encoded proteins mostly depend on the technology to deliver the coding DNA into the cells of interest. Since simple transfection methodology cannot be used in tissues, knockin or transgenic animals often with binary expression systems based on recombination (Cre-loxP) or transactivation technology need to be used. Here, the main determinant of protein expression is the specificity and strength of the promoter/enhancers. In case of binary expression systems, a ubiquitous promoter (eg, cytomegalovirus) can be used to optimize expression levels while cellular specificity is achieved by the control element driving Cre recombinase. Apart from transgenic animals, viral approaches can also be used either by injecting viral vector in the bloodstream[128] or by delivering vector intraluminally.[127] Here the combination of viral tropism and cell type specific promoters can help to yield expression in a subset of intestinal cells. For a comprehensive overview of genetic approaches that can be used to target specific cell types we refer to an excellent review by Huang and Zeng.[138]

solution.[127,128] An overview of possible strategies to deliver optical probes to intestinal tissues is summarized in Figure 3.

Conclusion and Future Perspectives

Remarkable improvements to live imaging, both in terms of equipment as well as in the design of optical probes, have made it an indispensable tool in physiology. In particular, the evolution of fluorescent probes is extraordinary, as it has generated a toolbox of genetically encoded proteins with which one can photo-manipulate as well as record from individual or entire networks of cells.

In the last 2 decades, these imaging techniques have proven instrumental in many discoveries in GI motility research. Unfortunately, the GI field has not adopted these techniques as eagerly as, for instance, CNS research. The reasons behind this are likely associated with issues inherent to imaging in the gut wall, and maybe also the cost of investing in an expensive microscopy set-up. The latter has been largely overcome, since it has become possible to record from bright probes such as Fluo-4 and GCaMPs with relatively cheap microscopy equipment. One problem of key importance is the fact that it has been extremely difficult to deliver, in flexible manner, foreign genetic material into cells residing in the gut wall. The underlying reason still remains unclear. Another drawback that is not specific to GI, but typical for the powerful genetic approaches, is that the rate at which new probes with slight alterations are published vastly exceeds the possibility to test them. Unfortunately, that seems to be the fate of this technology, and it will remain very crucial to select the correct version of the reporter, control elements and delivery route tuned to the need of the experiment.

Also in terms of equipment, interesting technology has been continually developed. Although not directly applicable to mammalian tissue because of scattering, it is noteworthy to mention here the single plane illumination techniques (SPIM) that allow imaging single planes at high speed with relatively low magnification. Mickoleit et al[129] recently succeeded using SPIM and reconstruction algorithms to make a full 3D film of the beating zebrafish heart. Interestingly, also an intravital microscopy technique has been developed that enables, via an abdominal imaging window, live imaging of epithelial crypt homeostasis in the intestinal mucosa.[130,131] Application of these techniques to monitor cellular activity of enteric neurons and other cell types in the gut would profoundly impact on our understanding of the in vivo function of these cells. In addition to imaging fluorescent labels, also label free techniques emerge (autofluorescence and second/third harmonic imaging). This methodology offers the advantage that labels can be omitted and circumvents possible artifacts arising from the fact that molecules of interest are usually labeled with an additional, often much larger, marker or fluorescent protein (~27 kD). The obvious disadvantage of label free techniques is that they mostly require pulsed IR lasers to penetrate deep enough into the tissue and exert their effect. Nonetheless, with those lasers, multiphoton excitation and second harmonic imaging of non-centrosymmetric biomolecules such as myosin and collagen becomes possible.[132]

The future challenge in using the currently available live imaging probes will be the careful design of specific driver and expression system pairs to deliver bright and fast optical probes to the correct cells in the desired time window. For equipment, the challenges mainly revolve around increasing the speed at which (especially neuronal signals) can be recorded, penetration depth, as well as finding a solution to match high resolution recordings to low magnification overview in order to maximally involve the cellular circuit of interest. However, such system, even if not perfect, should allow us to investigate in detail the dynamic interactions between different cell types in the intestinal wall: what neuronal subtypes connect functionally to each other? How do neurons and glia work together to tune activity? How do immune cells interact with the ENS and the epithelium to maintain intestinal homeostasis?

Acknowledgements

M.H. and W.B. are postdoctoral fellows of FWO. M.H. is an NHMRC fellow. Most imaging was performed on equipment obtained via Hercules foundation grants to PVB.

Supplementary Material

Note: To access the supplementary movie mentioned in this article, visit the online version of *Journal of Neurogastroenterology and Motility* at http://www.jnmjournal.org, and at doi: http://dx.doi.org/10.5056/jnm15096.

References

1. Furness JB. The enteric nervous system and neurogastroenterology. Nat Rev Gastroenterol Hepatol 2012;9:286-294.
2. Sanders KM, Ward SM, Koh SD. Interstitial cells: regulators of smooth muscle function. Physiol Rev 2014;94:859-907.

3. Boesmans W, Lasrado R, Vanden Berghe P, Pachnis V. Heterogeneity and phenotypic plasticity of glial cells in the mammalian enteric nervous system. Glia 2015;63:229-241.
4. Gulbransen BD, Sharkey KA. Novel functional roles for enteric glia in the gastrointestinal tract. Nat Rev Gastroenterol Hepatol 2012;9:625-632.
5. Mowat AM, Agace WW. Regional specialization within the intestinal immune system. Nat Rev Immunol 2014;14:667-685.
6. Bayliss WM, Starling EH. The movements and innervation of the small intestine. J Physiol 1899;24:99-143.
7. Fenno L, Yizhar O, Deisseroth K. The development and application of optogenetics. Annu Rev Neurosci 2011;34:389-412.
8. Hanani M. Lucifer yellow - an angel rather than the devil. J Cell Mol Med 2012;16:22-31.
9. Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ. Primary structure of the Aequorea victoria green-fluorescent protein. Gene 1992;111:229-233.
10. Nakai J, Ohkura M, Imoto K. A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. Nat Biotechnol 2001; 19:137-141.
11. Klarenbeek J, Jalink K. Detecting cAMP with an EPAC-based FRET sensor in single living cells. Methods Mol Biol 2014;1071: 49-58.
12. Betzig E. Proposed method for molecular optical imaging. Opt Lett 1995;20:237-239.
13. Dickson RM, Cubitt AB, Tsien RY, Moerner WE. On/off blinking and switching behaviour of single molecules of green fluorescent protein. Nature 1997;388:355-358.
14. Hell SW. Microscopy and its focal switch. Nat Methods 2009;6: 24-32.
15. Hell SW, Wichmann J. Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. Opt Lett 1994;19:780-782.
16. Choquet D. The 2014 Nobel Prize in Chemistry: a large-scale prize for achievements on the nanoscale. Neuron 2014;84:1116-1119.
17. D'Este E, Kamin D, Göttfert F, El-Hady A, Hell SW. STED nanoscopy reveals the ubiquity of subcortical cytoskeleton periodicity in living neurons. Cell Rep 2015;10:1246-1251.
18. Martens MA, Boesmans W, Vanden Berghe P. Calcium imaging at kHz frame rates resolves millisecond timing in neuronal circuits and varicosities. Biomed Opt Express 2014;5:2648-2661.
19. Michel K, Michaelis M, Mazzuoli G, Mueller K, Vanden Berghe P, Schemann M. Fast calcium and voltage-sensitive dye imaging in enteric neurones reveal calcium peaks associated with single action potential discharge. J Physiol 2011;589(Pt 24):5941-5947.
20. Neunlist M, Peters S, Schemann M. Multisite optical recording of excitability in the enteric nervous system. Neurogastroenterol Motil 1999;11:393-402.
21. Obaid AL, Koyano T, Lindstrom J, Sakai T, Salzberg BM. Spatiotemporal patterns of activity in an intact mammalian network with single-cell resolution: optical studies of nicotinic activity in an enteric plexus. J Neurosci 1999;19:3073-3093.
22. Chung K, Wallace J, Kim SY, et al. Structural and molecular interrogation of intact biological systems. Nature 2013;497:332-337.
23. Renier N, Wu Z, Simon DJ, Yang J, Ariel P, Tessier-Lavigne M. iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging. Cell 2014;159:896-910.
24. Fu YY, Peng SJ, Lin HY, Pasricha PJ, Tang SC. 3-D imaging and illustration of mouse intestinal neurovascular complex. Am J Physiol Gastrointest Liver Physiol 2013;304:G1-G11.
25. Liu YA, Chung YC, Pan ST, et al. 3-D imaging, illustration, and quantitation of enteric glial network in transparent human colon mucosa. Neurogastroenterol Motil 2013;25:e324-e338.
26. Cohen LB, Salzberg BM, Grinvald A. Optical methods for monitoring neuron activity. Annu Rev Neurosci 1978;1:171-182.
27. Ross WN, Salzberg BM, Cohen LB, et al. Changes in absorption, fluorescence, dichroism, and Birefringence in stained giant axons: : optical measurement of membrane potential. J Membr Biol 1977;33:141-183.
28. Hillsley K, Kenyon JL, Smith TK. Ryanodine-sensitive stores regulate the excitability of AH neurons in the myenteric plexus of guinea-pig ileum. J Neurophysiol 2000;84:2777-2785.
29. Vanden Berghe P, Kenyon JL, Smith TK. Mitochondrial Ca^{2+} uptake regulates the excitability of myenteric neurons. J Neurosci 2002; 22:6962-6971.
30. Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 2000;1:11-21.
31. Silver RA, Whitaker M, Bolsover SR. Intracellular ion imaging using fluorescent dyes: artefacts and limits to resolution. Pflugers Arch 1992;420:595-602.
32. Loew LM. Voltage-sensitive dyes: measurement of membrane potentials induced by DC and AC electric fields. Bioelectromagnetics 1992;(suppl 1):179-189.
33. Canepari M, Willadt S, Zecevic D, Vogt KE. Imaging inhibitory synaptic potentials using voltage sensitive dyes. Biophys J 2010;98:2032-2040.
34. Fairless R, Beck A, Kravchenko M, et al. Membrane potential measurements of isolated neurons using a voltage-sensitive dye. PLoS One 2013;8:e58260.
35. Akemann W, Mutoh H, Perron A, Rossier J, Knopfel T. Imaging brain electric signals with genetically targeted voltage-sensitive fluorescent proteins. Nat Methods 2010;7:643-649.
36. Looger LL, Griesbeck O. Genetically encoded neural activity indicators. Curr Opin Neurobiol 2012;22:18-23.
37. Mutoh H, Mishina Y, Gallero-Salas Y, Knöpfel T. Comparative performance of a genetically-encoded voltage indicator and a blue voltage sensitive dye for large scale cortical voltage imaging. Front Cell Neurosci 2015;9:147.
38. Kralj JM, Douglass AD, Hochbaum DR, Maclaurin D, Cohen AE. Optical recording of action potentials in mammalian neurons using a microbial rhodopsin. Nat Methods 2011;9:90-95.
39. Cao G, Platisa J, Pieribone VA, Raccuglia D, Kunst M, Nitabach MN. Genetically targeted optical electrophysiology in intact neural circuits. Cell 2013;154:904-913.
40. Hochbaum DR, Zhao Y, Farhi SL, et al. All-optical electrophysiology in mammalian neurons using engineered microbial rhodopsins. Nat Methods 2014;11:825-833.
41. Zou P, Zhao Y, Douglass AD, et al. Bright and fast multicoloured voltage reporters via electrochromic FRET. Nat Commun 2014;5: 4625.
42. Paredes RM, Etzler JC, Watts LT, Zheng W, Lechleiter JD. Chemical calcium indicators. Methods 2008;46:143-151.

43. Miyawaki A, Griesbeck O, Heim R, Tsien RY. Dynamic and quantitative Ca^{2+} measurements using improved cameleons. Proc Natl Acad Sci USA 1999;96:2135-2140.
44. Dombeck DA, Harvey CD, Tian L, Looger LL, Tank DW. Functional imaging of hippocampal place cells at cellular resolution during virtual navigation. Nat Neurosci 2010;13:1433-1440.
45. Huber D, Gutnisky DA, Peron S, et al. Multiple dynamic representations in the motor cortex during sensorimotor learning. Nature 2012;484:473-478.
46. Keller GB, Bonhoeffer T, Hubener M. Sensorimotor mismatch signals in primary visual cortex of the behaving mouse. Neuron 2012;74:809-815.
47. Tian L, Hires SA, Mao T, et al. Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nat Methods 2009;6:875-881.
48. Zariwala HA, Borghuis BG, Hoogland TM, et al. A Cre-dependent GCaMP3 reporter mouse for neuronal imaging in vivo. J Neurosci 2012;32:3131-3141.
49. Yamada Y, Mikoshiba K. Quantitative comparison of novel GCaMP-type genetically encoded Ca^{2+} indicators in mammalian neurons. Front Cell Neurosci 2012;6:41.
50. Akerboom J, Chen TW, Wardill TJ, et al. Optimization of a GCaMP calcium indicator for neural activity imaging. J Neurosci 2012;32:13819-13840.
51. Chen TW, Wardill TJ, Sun Y, et al. Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature 2013;499:295-300.
52. Ohkura M, Sasaki T, Sadakari J, et al. Genetically encoded green fluorescent Ca^{2+} indicators with improved detectability for neuronal Ca^{2+} signals. PLoS One 2012;7:e51286.
53. Akerboom J, Carreras Calderón N, Tian L, et al. Genetically encoded calcium indicators for multi-color neural activity imaging and combination with optogenetics. Front Mol Neurosci 2013;6:2.
54. Grienberger C, Konnerth A. Imaging calcium in neurons. Neuron 2012;73:862-885.
55. Li Z, Burrone J, Tyler WJ, Hartman KN, Albeanu DF, Murthy VN. Synaptic vesicle recycling studied in transgenic mice expressing synaptopHluorin. Proc Natl Acad Sci USA 2005;102:6131-6136.
56. Miesenböck G, De Angelis DA, Rothman JE. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature 1998;394:192-195.
57. Granseth B, Lagnado L. The role of endocytosis in regulating the strength of hippocampal synapses. J Physiol 2008;586(Pt 24):5969-5982.
58. Li Y, Tsien RW. pHTomato, a red, genetically encoded indicator that enables multiplex interrogation of synaptic activity. Nat Neurosci 2012;15:1047-1053.
59. Betz WJ, Bewick GS. Optical analysis of synaptic vesicle recycling at the frog neuromuscular junction. Science 1992;255:200-203.
60. Reynolds IJ. Mitochondrial membrane potential and the permeability transition in excitotoxicity. Ann N Y Acad Sci 1999;893:33-41.
61. d'Ydewalle C, Krishnan J, Chiheb DM, et al. HDAC6 inhibitors reverse axonal loss in a mouse model of mutant HSPB1-induced Charcot-Marie-Tooth disease. Nat Med 2011;17:968-974.
62. Vanden Berghe P, Hennig GW, Smith TK. Characteristics of intermittent mitochondrial transport in guinea pig enteric nerve fibers. Am J Physiol Gastrointest Liver Physiol 2004;286:G671-G682.
63. Misgeld T, Kerschensteiner M, Bareyre FM, Burgess RW, Lichtman JW. Imaging axonal transport of mitochondria in vivo. Nat Methods 2007;4:559-561.
64. Reiner A, Isacoff EY. The Brain Prize 2013: the optogenetics revolution Trends Neurosci 2013;36:557-560.
65. Boyden ES. A history of optogenetics: the development of tools for controlling brain circuits with light. F1000 Biol Rep 2011;3:11.
66. Zemelman BV, Lee GA, Ng M, Miesenbock G. Selective photostimulation of genetically chARGed neurons. Neuron 2002;33:15-22.
67. Lima SQ, Miesenbock G. Remote control of behavior through genetically targeted photostimulation of neurons. Cell 2005;121:141-152.
68. Nagel G, Szellas T, Huhn W, et al. Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proc Natl Acad Sci USA 2003;100:13940-13945.
69. Nagel G, Brauner M, Liewald JF, Adeishvili N, Bamberg E, Gottschalk A. Light activation of channelrhodopsin-2 in excitable cells of Caenorhabditis elegans triggers rapid behavioral responses. Curr Biol 2005;15:2279-2284.
70. Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K. Millisecond-timescale, genetically targeted optical control of neural activity. Nat Neurosci 2005;8:1263-1268.
71. Gunaydin LA, Yizhar O, Berndt A, Sohal VS, Deisseroth K, Hegemann P. Ultrafast optogenetic control. Nat Neurosci 2010;13:387-392.
72. Lin JY, Lin MZ, Steinbach P, Tsien RY. Characterization of engineered channelrhodopsin variants with improved properties and kinetics. Biophys J 2009;96:1803-1814.
73. Berndt A, Yizhar O, Gunaydin LA, Hegemann P, Deisseroth K. Bi-stable neural state switches. Nat Neurosci 2009;12:229-234.
74. Berndt A, Schoenenberger P, Mattis J, et al. High-efficiency channelrhodopsins for fast neuronal stimulation at low light levels. Proc Natl Acad Sci USA 2011;108:7595-7600.
75. Zhang F, Prigge M, Beyrière F, et al. Red-shifted optogenetic excitation: a tool for fast neural control derived from Volvox carteri. Nat Neurosci 2008;11:631-633.
76. Gradinaru V, Thompson KR, Deisseroth K. eNpHR: a Natronomonas halorhodopsin enhanced for optogenetic applications. Brain Cell Biol 2008;36:129-139.
77. Liewald JF, Brauner M, Stephens GJ, et al. Optogenetic analysis of synaptic function. Nat Methods 2008;5:895-902.
78. Sohal VS, Zhang F, Yizhar O, Deisseroth K. Parvalbumin neurons and gamma rhythms enhance cortical circuit performance. Nature 2009;459:698-702.
79. Gradinaru V, Mogri M, Thompson KR, Henderson JM, Deisseroth K. Optical deconstruction of parkinsonian neural circuitry. Science 2009;324:354-359.
80. Kravitz AV, Freeze BS, Parker PR, et al. Regulation of parkinsonian motor behaviours by optogenetic control of basal ganglia circuitry. Nature 2010;466:622-626.
81. Depuy SD, Kanbar R, Coates MB, Stornetta RL, Guyenet PG. Control of breathing by raphe obscurus serotonergic neurons in mice. J Neurosci 2011;31:1981-1990.
82. Hagglund M, Dougherty KJ, Borgius L, Itohara S, Iwasato T, Kiehn O. Optogenetic dissection reveals multiple rhythmogenic modules underlying locomotion. Proc Natl Acad Sci USA 2013;110:11589-11594.
83. Johansen JP, Hamanaka H, Monfils MH, et al. Optical activation of

lateral amygdala pyramidal cells instructs associative fear learning. Proc Natl Acad Sci USA 2010;107:12692-12697.

84. Lin JY. A user's guide to channelrhodopsin variants: features, limitations and future developments. Exp Physiol 2011;96:19-25.
85. Avetisyan M, Schill EM, Heuckeroth RO. Building a second brain in the bowel. J Clin Invest 2015;125:899-907.
86. Sasselli V, Pachnis V, Burns AJ. The enteric nervous system. Dev Biol 2012;366:64-73.
87. Yntema CL, Hammond WS. The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. J Comp Neurol 1954;101:515-541.
88. Kulesa PM, Fraser SE. Neural crest cell dynamics revealed by time-lapse video microscopy of whole embryo chick explant cultures. Dev Biol 1998;204:327-344.
89. Kulesa PM, Fraser SE. In ovo time-lapse analysis of chick hindbrain neural crest cell migration shows cell interactions during migration to the branchial arches. Development 2000;127:1161-1172.
90. Carmona-Fontaine C, Matthews HK, Kuriyama S, et al. Contact inhibition of locomotion in vivo controls neural crest directional migration. Nature 2008;456:957-961.
91. Druckenbrod NR, Epstein ML. The pattern of neural crest advance in the cecum and colon. Dev Biol 2005;287:125-133.
92. Wang X, Chan AK, Sham MH, Burns AJ, Chan WY. Analysis of the sacral neural crest cell contribution to the hindgut enteric nervous system in the mouse embryo. Gastroenterology 2011;141:992-1002, e1-e6.
93. Young HM, Bergner AJ, Anderson RB, et al. Dynamics of neural crest-derived cell migration in the embryonic mouse gut. Dev Biol 2004;270:455-473.
94. Adam V. Phototransformable fluorescent proteins: which one for which application? Histochem Cell Biol 2014;142:19-41.
95. Ando R, Hama H, Yamamoto-Hino M, Mizuno H, Miyawaki A. An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. Proc Natl Acad Sci USA 2002; 99:12651-12656.
96. Tsutsui H, Karasawa S, Shimizu H, Nukina N, Miyawaki A. Semi-rational engineering of a coral fluorescent protein into an efficient highlighter. EMBO Rep 2005;6:233-238.
97. Gurskaya NG, Verkhusha VV, Shcheglov AS, et al. Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. Nat Biotechnol 2006;24:461-465.
98. Zhou XX, Lin MZ. Photoswitchable fluorescent proteins: ten years of colorful chemistry and exciting applications. Curr Opin Chem Biol 2013;17:682-690.
99. Nishiyama C, Uesaka T, Manabe T, et al. Trans-mesenteric neural crest cells are the principal source of the colonic enteric nervous system. Nat Neurosci 2012;15:1211-1218.
100. Young HM, Bergner AJ, Simpson MJ, et al. Colonizing while migrating: how do individual enteric neural crest cells behave? BMC Biol 2014;12:23.
101. Hao MM, Boesmans W, Van den Abbeel V, et al. Early emergence of neural activity in the developing mouse enteric nervous system. J Neurosci 2011;31:15352-15361.
102. Foong JP, Hirst CS, Hao MM, et al. Changes in nicotinic neurotransmission during enteric nervous system development. J Neurosci 2015;35:7106-7115.
103. Bayguinov PO, Hennig GW, Smith TK. Calcium activity in different classes of myenteric neurons underlying the migrating motor complex in the murine colon. J Physiol 2010;588(Pt 3):399-421.
104. Heredia DJ, Dickson EJ, Bayguinov PO, Hennig GW, Smith TK. Colonic elongation inhibits pellet propulsion and migrating motor complexes in the murine large bowel. J Physiol 2010;588(Pt 15): 2919-2934.
105. Smith TK, Park KJ, Hennig GW. Colonic migrating motor complexes, high amplitude propagating contractions, neural reflexes and the importance of neuronal and mucosal serotonin. J Neurogastroenterol Motil 2014;20:423-446.
106. Mazzuoli G, Schemann M. Multifunctional rapidly adapting mechanosensitive enteric neurons (RAMEN) in the myenteric plexus of the guinea pig ileum. J Physiol 2009;587(Pt 19):4681-4694.
107. Schemann M, Mazzuoli G. Multifunctional mechanosensitive neurons in the enteric nervous system. Auton Neurosci 2010;153:21-25.
108. Boesmans W, Martens MA, Weltens N, et al. Imaging neuron-glia interactions in the enteric nervous system. Front Cell Neurosci 2013; 7:183.
109. Gulbransen BD, Sharkey KA. Purinergic neuron-to-glia signaling in the enteric nervous system. Gastroenterology 2009;136:1349-1358.
110. Broadhead MJ, Bayguinov PO, Okamoto T, Heredia DJ, Smith TK. Ca^{2+} transients in myenteric glial cells during the colonic migrating motor complex in the isolated murine large intestine. J Physiol 2012;590(Pt 2):335-350.
111. McClain JL, Grubisic V, Fried D, et al. Ca^{2+} responses in enteric glia are mediated by connexin-43 hemichannels and modulate colonic transit in mice. Gastroenterology 2014;146:497-507, e1.
112. Huizinga JD, Chen JH, Zhu YF, et al. The origin of segmentation motor activity in the intestine. Nat Commun 2014;5:3326.
113. Singh RD, Gibbons SJ, Saravanaperumal SA, et al. Ano1, a Ca^{2+}-activated Cl^{-} channel, coordinates contractility in mouse intestine by Ca^{2+} transient coordination between interstitial cells of Cajal. J Physiol 2014;592(Pt 18):4051-4068.
114. Baker SA, Hennig GW, Ward SM, Sanders KM. Temporal sequence of activation of cells involved in purinergic neurotransmission in the colon. J Physiol2015;593:1945-1963.
115. Travis L, Spencer NJ. Imaging stretch-activated firing of spinal afferent nerve endings in mouse colon. Front Neurosci 2013;7:179.
116. Matteoli G, Gomez-Pinilla PJ, Nemethova A, et al. A distinct vagal anti-inflammatory pathway modulates intestinal muscularis resident macrophages independent of the spleen. Gut 2014;63:938-948.
117. Nemethova A, Michel K, Gomez-Pinilla PJ, Boeckxstaens GE, Schemann M. Nicotine attenuates activation of tissue resident macrophages in the mouse stomach through the beta2 nicotinic acetylcholine receptor. PLoS One 2013;8:e79264.
118. Schemann M, Camilleri M. Functions and imaging of mast cell and neural axis of the gut. Gastroenterology 2013;144:698-704, e4.
119. Buhner S, Braak B, Li Q, et al. Neuronal activation by mucosal biopsy supernatants from irritable bowel syndrome patients is linked to visceral sensitivity. Exp Physiol 2014;99:1299-1311.
120. Buhner S, Li Q, Vignali S, et al. Activation of human enteric neurons by supernatants of colonic biopsy specimens from patients with irritable bowel syndrome. Gastroenterology 2009;137:1425-1434.
121. Cirillo C, Tack J, Vanden Berghe P. Nerve activity recordings in routine human intestinal biopsies. Gut 2013;62:227-235.
122. Boesmans W, Gomes P, Janssens J, Tack J, Vanden Berghe P.

Brain-derived neurotrophic factor amplifies neurotransmitter responses and promotes synaptic communication in the enteric nervous system. Gut 2008;57:314-322.

123. Vanden Berghe P, Klingauf J. Spatial organization and dynamic properties of neurotransmitter release sites in the enteric nervous system. Neuroscience 2007;145:88-99.

124. Vanden Berghe P, Tack J, Boesmans W. Highlighting synaptic communication in the enteric nervous system. Gastroenterology 2008;135:20-23.

125. Boesmans W, Ameloot K, van den Abbeel V, Tack J, Vanden Berghe P. Cannabinoid receptor 1 signalling dampens activity and mitochondrial transport in networks of enteric neurones. Neurogastroenterol Motil 2009;21:958, e77.

126. Gallego D, Vanden Berghe P, Farré R, Tack J, Jiménez M. P2Y1 receptors mediate inhibitory neuromuscular transmission and enteric neuronal activation in small intestine. Neurogastroenterol Motil 2008;20:159-168.

127. Benskey MJ, Kuhn NC, Galligan JJ, et al. Targeted gene delivery to the enteric nervous system using AAV: a comparison across serotypes and capsid mutants. Mol Ther 2015;23:488-500.

128. Gombash SE, Cowley CJ, Fitzgerald JA, et al. Intravenous AAV9 efficiently transduces myenteric neurons in neonate and juvenile mice. Front Mol Neurosci 2014;7:81.

129. Mickoleit M, Schmid B, Weber M, et al. High-resolution reconstruction of the beating zebrafish heart. Nat Methods 2014;11:919-922.

130. Ritsma L, Ellenbroek SI, Zomer A, et al. Intestinal crypt homeostasis revealed at single-stem-cell level by in vivo live imaging. Nature 2014;507:362-365.

131. Ritsma L, Steller EJ, Ellenbroek SI, Kranenburg O, Borel Rinkes IH, van Rheenen J. Surgical implantation of an abdominal imaging window for intravital microscopy. Nat Protoc 2013;8:583-594.

132. Birk JW, Tadros M, Moezardalan K, et al. Second harmonic generation imaging distinguishes both high-grade dysplasia and cancer from normal colonic mucosa. Dig Dis Sci 2014;59:1529-1534.

133. Vanden Berghe P, Missiaen L, Bellon E, Vanderwinden JM, Janssens J, Tack J. Free cytosolic Ca^{2+} recordings from myenteric neurones in multilayer intestinal preparations. Neurogastroenterol Motil 2001;13:493-502.

134. Stevens RJ, Publicover NG, Smith TK. Induction and organization of Ca^{2+} waves by enteric neural reflexes. Nature 1999;399:62-66.

135. Hennig GW, Smith CB, O'Shea DM, Smith TK. Patterns of intracellular and intercellular Ca^{2+} waves in the longitudinal muscle layer of the murine large intestine in vitro. J Physiol2002;543(Pt 1):233-253.

136. Bisschops R, Vanden Berghe P, Sarnelli G, Janssens J, Tack J. CRF-induced calcium signaling in guinea pig small intestine myenteric neurons involves CRF-1 receptors and activation of voltage-sensitive calcium channels. Am J Physiol Gastrointest Liver Physiol 2006;290:G1252-G1260.

137. Gulbransen BD, Bains JS, Sharkey KA. Enteric glia are targets of the sympathetic innervation of the myenteric plexus in the guinea pig distal colon. J Neurosci 2010;30:6801-6809.

138. Huang ZJ, Zeng H. Genetic approaches to neural circuits in the mouse. Annu Rev Neurosci 2013;36:183-215.