Published in final edited form as:

Neurogastroenterol Motil. 2018 September ; 30(9): e13435. doi:10.1111/nmo.13435.

Improved Imaging of Zebrafish Motility

Adam Rich

The College at Brockport, SUNY, 350 New Campus Drive, Brockport, NY 14420 USA, Telephone: 585-395-5740

Abstract

Zebrafish larvae are transparent and the entire gastrointestinal (GI) tract is easily visualized. Application of a new image analysis technique is reported in this issue of *Neurogastroenterology and Motility*¹. The technique quantifies movement in images collected in a timed sequence, and characterizes smooth muscle contractions based on contraction distance and frequency. The technique also reports the contraction amplitude, or the distance moved. This technique, as well as current spatiotemporal mapping techniques, are essential tools enabling characterization of GI motility patterns in intact physiological settings. Advances and development of transgenic zebrafish that lack pigmentation, with calcium reporters expressed in specific cell types, or with inactivation of specific genes contribute to our understanding of the generation, and regulation of GI motility at the molecular, cellular, and systemic level. Finally, development of chambers that immobilize zebrafish larvae for long-duration imaging will contribute to our technique toolbox, and will provide an increased experimental throughput.

Abbreviated abstract

This minireview reports a new imaging technique suitable for quantifying gastrointestinal motility patterns in transparent zebrafish larvae. Advancements in transgenic zebrafish and chamber design applicable to motility measurements are described. Current techniques for Code for the technique is publicly available.

Keywords

zebrafish; velocimetry; spatiotemporal maps; gastrointestinal motility

Generation and regulation of coordinated muscular contractions of the gastrointestinal (GI) tract results from complex interactions between smooth muscle cells, enteric neurons and interstitial cells of Cajal. Regulators external to the GI tract, such as the autonomic nervous

system, mast and macrophage cells, as well as endocrine factors match GI motility patterns to other body systems². Luminal contents and microbiota can also influence motility patterns³. This level of complexity supports efficient nutrient absorption and waste elimination, and helps to prevent damage to the intestinal epithelial barrier thereby preventing infection⁴. Unraveling control of GI motility is difficult because control mechanisms overlap, are interrelated, and in combination result in a robust system that remains functional despite injury^{5,6}. This complexity is an excellent rationale for a reductionist approach, isolating cells or knocking out single genes, to better understand regulatory mechanisms in isolation. The complexity also underscores the relevance for in-vivo approaches in unperturbed animals to better understand the complete system underlying generation and regulation of GI motor patterns, and to not miss potential interactions between regulatory pathways. Imaging GI motility in transparent zebrafish larvae provides an opportunity to examine GI motor function and regulation in an anesthetized, but otherwise unperturbed animal. Developing treatments to restore GI motility in the presence of a regulatory deficit, possible by removing ICC or enteric neurons, requires in-vivo models. Ultimately, our ability to restore GI motility clinically requires a deep understanding of intact and damaged GI systems. This mini-review will describe development of the zebrafish model for GI motility, focusing on imaging performed in developing larvae.

An article published in this issue of *Neurogastroenterology and Motility* entitled *Image velocimetry and spectral analysis* characterizes larval zebrafish gut motility using a new image analysis method that measures contraction frequency, amplitude, velocity, and distance¹. Smooth muscle contractions that result in ring-like lumen constriction and shortening of the digestive tract are quantified from time lapse images and presented as quantitative spatiotemporal maps (QSTMaps, see figure 1)¹. Propagating contractions are easily observed in the QSTMap, and the repeating pattern indicates that contractions are organized. Contraction cycles occur slightly more than 2 per minute, or ~25 seconds between contractions. QSTMaps also reveal the correlation between circular and longitudinal smooth muscle contractions, and show circular contractions leading longitudinal contractions by 1 or 2 seconds (figure 2). Application of acetylcholine increased contraction frequency and contraction amplitude in 6 day old zebrafish larvae, as expected, contributing to validation of this technique⁷. Feeding larvae for 2 days increased contraction frequency and amplitude when compared to fasted larvae, also as expected^{8,9}. Interestingly, feeding appears to increase the variability in contraction amplitude. It is important to know that for this analysis contraction amplitude refers to the overall shortening of smooth muscle, and it does not measure contraction strength¹.

Looking back, characterization of GI motility took the straightforward approach of counting spontaneous contractions and reporting contraction frequency in zebrafish larvae¹⁰. These studies anesthetized and immobilized larvae using warmed agarose with tricaine (0.1g/L), and quickly positioning larvae for an optimal lateral view before the agarose solidifies, and covering with a layer of embryo medium^{7,10}. This remains the most common immobilization technique¹¹. Time lapse imaging collects images and contraction frequency at a specific point along the GI tract is determined by simply watching images playback at a high frame rate. Holmberg and co-workers reported 0.97 ± 0.2 contractions/min in 6–8 dpf larvae¹². Spatiotemporal mapping techniques were applied later to zebrafish GI motility by

Author Manuscript

Holmberg, Olsson, and Hennig where they showed the role of enteric neurons using tetrodotoxin¹³. They reported contraction frequency of 1.05 ± 0.09 contractions/minute, contraction distance as $720 \pm 33 \mu\text{m}$, and contraction velocity of $28 \pm 4 \mu\text{m/sec}$ ¹³. Inhibition of enteric neural activity using tetrodotoxin decreased both contraction frequency and contraction distance by ~50%. Spatiotemporal maps for this work averages pixels in the vertical axis over a region of interest drawn over the intestine beginning after the swim bladder. Spatiotemporal maps are constructed using the averages, and each horizontal line in the map corresponds to a single timepoint. This spatiotemporal analysis technique was an important advance and provided quantitative measures to characterize GI motility for the first time.

Author Manuscript

ICC were first identified in zebrafish using anti-Kit antibodies¹⁴. The KIT protein is a receptor tyrosine kinase and is necessary for ICC development in humans, mice, and zebrafish^{15–17}. The functional role for ICC in zebrafish was shown using imatinib mesylate to inhibit KIT function, and therefore preventing ICC development¹⁷. Spatiotemporal maps showed that imatinib mesylate-treated larvae had fewer spontaneous coordinated contractions, and when contractions occurred they were shorter distance and slower propagation velocity. A second advance for larvae positioning also contributed to this analysis using an ethylene propylene tube¹⁸. Drawing the larvae into the tube, in agarose, provides a simple and convenient method to easily rotate larvae to optimize lateral imaging position 7 dpf larvae with little to no distortion.

Author Manuscript

Ganz and coworkers describe a novel analysis technique using particle image velocimetry to measure gut motility. Particle image velocimetry is a technique that is more commonly used to characterize fluid flow by tracking movement of particles suspended in the fluid from frame to frame¹⁹. One intriguing advantage of the technique is that it quantifies movement of each pixel in each frame, which is visualized using vectors superimposed on the image data (see Ganz supplemental movie). Using this analysis it is feasible to quantify and correlate movement of luminal contents with smooth muscle contractions. Software developed for this analysis is publicly available allowing the possibility for implementation of this methodology. The authors describe the complex analysis method succinctly, and the end product is a QSTMap which displays GI motility in a straightforward manner that allows coordinated patterns to be recognized. A custom built differential interference contrast microscope that allows simultaneous light sheet fluorescence microscopy and differential interference contrast microscopy (DIC) enhances the image data²⁰. DIC imaging excels at highlighting edges between cells or between the lumen and the mucosa.

Author Manuscript

Resolution for QSTMaps is determined by the magnification and the image frame rate. Images were collected with a sCMOS camera, which excels at fast imaging and provides high dynamic range, and a 40X objective providing a frame size of approximately $400 \mu\text{m}$ (see Ganz, supplemental movie 1). This provides a resolution of approximately $0.2 \mu\text{m/pixel}$. For comparison, the GI tract, measured from the intestinal bulb to the anus, of a 7 dpf zebrafish is approximately 1.5 cm (see Ganz, Figure 1A). The STMaps therefore focuses on an approximately 25% of the entire GI tract. At this resolution characterization of interactions between individual smooth muscle cells may be possible. The external muscular layers of the GI tract function as a syncytium and are regulated by ICC and enteric neurons.

Examining communication between ICC and enteric neurons or ICC and smooth muscle in vivo is an exciting possibility. For comparison by size, myenteric ICC cell bodies in the zebrafish GI tract are approximately 25 μm apart with stellate processes extending to neighboring ICC and smooth muscle cells (Figure 1).

Increased availability of imaging tools to analyze movement will enable correlations between GI motor patterns and specific roles between individual cells to be realized. A rapidly expanding list of available transgenic zebrafish label specific cell types and specific signaling mechanisms will also accelerate discovery. For example, macrophage influence gastric emptying is diabetic mice²¹. Using a transgenic zebrafish line expressing fluorescently labeled macrophage will allow movement of macrophage cells within the smooth muscle layers of the GI tract to be tracked in-vivo, while simultaneously measuring GI motility²². Pigment cells begin to develop at 2 days post fertilization, well before the time when the GI tract develops at 4 days post fertilization, and the pigment cells interfere with imaging. Two fish lines, *casper* and *crystal* lack pigmentation^{23, 24}. Genetically coded calcium indicators driven by tissue-specific promoters enable signaling activity in specific cell populations to be collected during GI motility²⁵. Interactions between the intestinal contents and GI smooth muscles can also be measured. For example, movement of rod-shaped *Vibrio cholera* bacteria in zebrafish GI tract, with movements tracked using DIC and velocimetry, suggest that it is possible to characterize feedback between the GI tract and the intestinal contents^{20, 26}. A connection between intestinal microbial populations and GI motility has already been shown in a simplified system with just 2 microbial species established in a zebrafish larva GI tract^{27, 28}.

GI motility patterns change over time and understanding how changing patterns is regulated is an important goal. Image analysis over a time period of 70 seconds, as shown in the accompany article, or over several minutes as shown in other publications, show only a few propagating contractions^{13, 17}. Contractions appear to be ring-like propagating contractions, as observed by a distinct streak in the spatiotemporal maps, or as much smaller ripples that may not propagate and may perform a mixing function. Propagating contractions analyzed by Ganz et al may be most similar to the migrating motor complex in humans, a cyclic motility pattern observed during fasting²⁹. Characterizing transitions between motility patterns requires image sequences spanning longer time periods but immobilization in agarose or polyethylene tubes sometimes results in deteriorating larvae health when recordings last more than 20 minutes. Many platforms for medium throughput compound testing on zebrafish embryo have been described^{30, 31}. We have experimented with a Neofluidics millifluidic chamber that allows perfusion and can hold multiple larvae for several hours. One spatiotemporal map resulting from a 60 minute recording (4 frames/second resulting in a 1.64GB file size) is shown in figure 2. The middle and distal intestine show consistent propagating contractions but the intestinal bulb shows changing contraction patterns. GI motor patterns can be analyzed from image time series while simultaneously measuring GI transit time, or signaling activity in select groups of cells. The chamber enables positioning of multiple embryos with perfusion, and therefore is suitable for medium-throughput compound testing on zebrafish GI motility at the whole organ level, or at cellular resolution.

Improved image analysis, chamber design, and availability of transgenic zebrafish combine to accelerate our understanding of the regulation of GI motility. Basic work to better understand development of ICC and enteric neurons remains to be done, and a better understanding of the similarities and differences between 7 day old and adult GI tracts of this in-vivo vertebrate model system will contribute to understanding regulatory processes. Physiologists are joining developmental biologists, taking advantage of the optical clarity and real-time imaging made possible by transparent zebrafish larvae.

Acknowledgments

I thank Grant Hennig for Volumetry software and useful discussions and Jon Sleeper for the data shown in Figure 2. Research in this publication was supported by NIH grant DK071588 and by a SUNY Research Foundation Collaboration grant.

References

1. Ganz J. Image velocimetry and spectral analysis enable quantitative characterization of larval zebrafish gut motility. *Neurogastroenterol Motil.* 2018
2. Sanders KM, Koh SD, Ro S, Ward SM. Regulation of gastrointestinal motility—insights from smooth muscle biology. *Nature reviews Gastroenterology & hepatology.* 2012; 9:633–45. [PubMed: 22965426]
3. Carabotti M, Scirocco A, Maselli MA, Severi C. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Annals of Gastroenterology : Quarterly Publication of the Hellenic Society of Gastroenterology.* 2015; 28:203–9.
4. Groschwitz KR, Hogan SP. Intestinal Barrier Function: Molecular Regulation and Disease Pathogenesis. *The Journal of allergy and clinical immunology.* 2009; 124:3–22. [PubMed: 19560575]
5. Huizinga JD, Lammers WJ. Gut peristalsis is governed by a multitude of cooperating mechanisms. *American Journal of Physiology-Gastrointestinal and Liver Physiology.* 2009; 296:G1–G8. [PubMed: 18988693]
6. Farrugia G. Interstitial cells of Cajal in health and disease. *Neurogastroenterol Motil.* 2008; 20(Suppl 1):54–63. [PubMed: 18402642]
7. Holmberg A, Schwerte T, Pelster B, Holmgren S. Ontogeny of the gut motility control system in zebrafish *Danio rerio* embryos and larvae. *J Exp Biol.* 2004; 207:4085–94. [PubMed: 15498954]
8. Maljaars J, Peters HP, Masclee AM. Review article: The gastrointestinal tract: neuroendocrine regulation of satiety and food intake. *Alimentary pharmacology & therapeutics.* 2007; 26(Suppl 2): 241–50. [PubMed: 18081667]
9. Miller KE, Bajzer Z, Hein SS, Phillips JE, Syed S, Wright AM, et al. High temporal resolution gastric emptying breath tests in mice. *Neurogastroenterol Motil.* 2018:e13333. [PubMed: 29575442]
10. Holmberg A, Schwerte T, Fritsche R, Pelster B, Holmgren S. Development of spontaneous activity in the zebrafish gut. *J Fish Biol.* 2003; 63:318–31.
11. Shi Y, Zhang Y, Zhao F, Ruan H, Huang H, Luo L, et al. Acetylcholine serves as a derepressor in Loperamide-induced Opioid-Induced Bowel Dysfunction (OIBD) in zebrafish. *Scientific reports.* 2014; 4:5602. [PubMed: 24998697]
12. Holmqvist B. The early ontogeny of neuronal nitric oxide synthase systems in the zebrafish. *Journal of Experimental Biology.* 2004; 207:923–35. [PubMed: 14766951]
13. Holmberg A, Olsson C, Hennig GW. TTX-sensitive and TTX-insensitive control of spontaneous gut motility in the developing zebrafish (*Danio rerio*) larvae. *J Exp Biol.* 2007; 210:1084–91. [PubMed: 17337720]
14. Rich A, Leddon S, Hess S, Gibbons S, Miller S, Xu X, et al. Kit-like immunoreactivity in the zebrafish gastrointestinal tract reveals putative ICC. *Developmental Dynamics.* 2007; 236:903–11. [PubMed: 17295318]

15. Maeda H, Yamagata A, Nishikawa S, Yoshinaga K, Kobayashi S, Nishi K. Requirement of c-kit for development of intestinal pacemaker system. *Development*. 1992; 116:369–75. [PubMed: 1283735]
16. Ward SM, Burns AJ, Torihashi S, Sanders KM. Mutation of the proto-oncogene c-kit blocks development of interstitial cells and electrical rhythmicity in murine intestine. *The Journal of physiology*. 1994; 480:91–7. [PubMed: 7853230]
17. Rich A, Gordon S, Brown C, Gibbons SJ, Schaefer K, Hennig G, et al. Kit signaling is required for development of coordinated motility patterns in zebrafish gastrointestinal tract. *Zebrafish*. 2013; 10:154–60. [PubMed: 23297728]
18. Petzold AM, Bedell VM, Boczek NJ, Essner JJ, Balciunas D, Clark KJ, et al. SCORE imaging: specimen in a corrected optical rotational enclosure. *Zebrafish*. 2010; 7:149–54. [PubMed: 20528262]
19. Ryerson WG, Schwenk K. A simple, inexpensive system for digital particle image velocimetry (DPIV) in biomechanics. *Journal of experimental zoology Part A, Ecological genetics and physiology*. 2012; 317:127–40.
20. Baker RP, Taormina MJ, Jemielita M, Parthasarathy R. A Combined Light Sheet Fluorescence and Differential Interference Contrast Microscope for Live Imaging of Multicellular Specimens. *Journal of microscopy*. 2015; 258:105–12. [PubMed: 25611324]
21. Cipriani G, Gibbons SJ, Miller KE, Yang DS, Terhaar ML, Eisenman ST, et al. Change in Populations of Macrophages Promotes Development of Delayed Gastric Emptying in Mice. *Gastroenterology*. 2018; 154:2122–36e12. [PubMed: 29501441]
22. Torraca V, Masud S, Spaink HP, Meijer AH. Macrophage-pathogen interactions in infectious diseases: new therapeutic insights from the zebrafish host model. *Disease models & mechanisms*. 2014; 7:785–97. [PubMed: 24973749]
23. Antinucci P, Hindges R. A crystal-clear zebrafish for in vivo imaging. *Scientific reports*. 2016; 6:29490. [PubMed: 27381182]
24. White RM, Sessa A, Burke C, Bowman T, LeBlanc J, Ceol C, et al. Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell stem cell*. 2008; 2:183–9. [PubMed: 18371439]
25. Renninger SL, Orger MB. Two-photon imaging of neural population activity in zebrafish. *Methods*. 2013; 62:255–67. [PubMed: 23727462]
26. Ganz J, Melancon E, Eisen JS. Chapter 5 - Zebrafish as a model for understanding enteric nervous system interactions in the developing intestinal tract. In: Detrich HW, Westerfield M, Zon LI, editors *Methods in Cell Biology*. Academic Press; 2016. 139–64.
27. Rolig AS, Mittge EK, Ganz J, Troll JV, Melancon E, Wiles TJ, et al. The enteric nervous system promotes intestinal health by constraining microbiota composition. *PLoS biology*. 2017; 15:e2000689. [PubMed: 28207737]
28. Wiles TJ, Jemielita M, Baker RP, Schlomann BH, Logan SL, Ganz J, et al. Host gut motility promotes competitive exclusion within a model intestinal microbiota. *PLoS biology*. 2016; 14:e1002517. [PubMed: 27458727]
29. Deloof E, Janssen P, Depoortere I, Tack J. The migrating motor complex: control mechanisms and its role in health and disease. *Nat Rev Gastroenterol Hepatol*. 2012; 9:271–85. [PubMed: 22450306]
30. Pardo-Martin C, Chang T-Y, Koo BK, Gilleland CL, Wasserman SC, Yanik MF. High-throughput in vivo vertebrate screening. *Nature Methods*. 2010; 7:634. [PubMed: 20639868]
31. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. *Nature Reviews Genetics*. 2007; 8:353.

Figure 1.

Dual staining shows the relative positions of ICC and enteric neurons in adult zebrafish (A, B), and smooth muscle and enteric neurons in larvae (C). Anti anoctamin 1 antibody (magenta) identifies ICC and anti tubulin antibody (green) identifies enteric neurons. Stellate ICC (A) are located between circular and smooth muscle layers. Bipolar ICC (B) are located within the circular smooth muscle layer close to the mucosal border. Close appositions between ICC and enteric neurons are observed for both types of ICC. Anti SM 22 antibody (magenta) identifies smooth muscle cells and anti-tubulin identifies enteric neurons (green, C) in the intestinal bulb. Scale bars are 50 μm .

Figure 2.

Spatiotemporal map lasting 1 hour. 8 days post fertilization larvae was immobilized in a Neofluidics perfusion chamber and images were collected at 4 frames/second. The top panel shows the region of interest extending from the intestinal bulb to the anus and the bottom panel shows the spatiotemporal map for 60 minutes. Consistent propagating contractions are apparent in the mid to distal intestine for the duration of this recording. A different pattern is observed in the intestinal bulb, with occasional changes in frequency and appearance.