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Whole-animal Imaging, Gene Function, and the Zebrafish Phenome Project

Keith C. Cheng[1,†], **Xuying Xin**[1], **Darin Clark**[1,2], and **Patrick La Riviere**[3]

[1]Jake Gittlen Cancer Research Foundation and Division of Experimental Pathology, Penn State Hershey College of Medicine, Hershey, PA 17033

[3]Department of Radiology, University of Chicago, Chicago, IL 60637

Abstract

Imaging can potentially make a major contribution to the zebrafish phenome project, which will probe the functions of vertebrate genes through the generation and phenotyping of mutants. Imaging of whole animals at different developmental stages through adulthood will be used to infer biological function. Cell resolutions will be required to identify cellular mechanism and to detect a full range of organ effects. Light-based imaging of live zebrafish embryos is practical only up to ~2 days of development, due to increasing pigmentation and diminishing tissue lucency with age. The small size of the zebrafish makes possible whole-animal imaging at cell resolutions by histology and micron-scale tomography (microCT). The histological study of larvae is facilitated by the use of arrays, and histology's standard use in the study of human disease enhances its translational value. Synchrotron microCT with X-rays of moderate energy (10-25 keV) is unimpeded by pigmentation or the tissue thicknesses encountered in zebrafish of larval stages and beyond, and is well-suited to detecting phenotypes that may require 3D modeling. The throughput required for this project will require robotic sample preparation and loading, increases in the dimensions and sensitivity of scintillator and CCD chips, increases in computer power, and the development of new approaches to image processing, segmentation, and quantification.

Introduction: The Zebrafish Phenome Project

A fundamental principle of genetics is that the structural, physiological or behavioral *phenotype* of an organism with a deficiency in a specific gene function informs us about the gene's function(s). Phenotyping an organism is limited by the available methods, typically limited to the focus of the individual scientist and complicated by the often multiple functions of any given gene, as illustrated by pleiotropy [1, 2, 3, 4, 5]. The *totality of possible phenotypes*, the *phenome* [6• 7•] is a conceptual construct that, together with imaging, comprises the focus of this review. To address the functions of the more than 20,000 vertebrate genes, and inspired by the development of phenomics [8•, 9, 10,11, 12], the international zebrafish community is planning a "Zebrafish Phenome Project[1]". Multiple

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†Corresponding Author. Tel 717-531-5635. Fax 717-531-5634, kcheng76@gmail.com.

[2]Present address: Department of Biomedical Engineering, Duke University, Durham, NC 27708, dpclark@gmail.com

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[1]The term "zebrafish phenome project " (http://www.blsmeetings.net/zebrafish/) is convenient. To distinguish phenotypes associated with gene deficiencies from those caused by diseases or chemicals, "zebrafish *genetic* phenome project", while less practical, is more precise.

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methods of mutagenesis [13] will be used toward the goal of producing at least one mutant allele per gene in the next five years, and the mutants will then be phenotyped. At a March 2010 meeting sponsored by Sanger Center and the National Human Genome Research Institute at NIH, the zebrafish research community discussed potential phenotyping assays. These included physiological and behavioral assays, as well as fluorescence-based morphological assays that require live animals. Here, we focus on the assessment of phenotypes that change the morphological features of cells, tissues, and organs in whole, fixed zebrafish that are too pigmented and too thick for 3D imaging at cell resolutions using light. The issues discussed included methods of imaging, reproducibility of assays, and throughput of mutant production. Imaging features ideal for this project are listed in Table 1.

Zebrafish as a model for vertebrate biology and human disease

The zebrafish is a vertebrate model with compelling experimental features, including embryonic transparency, fecundity, and a sophisticated genetic tool box including genetic screens, morpholino knock-downs of virtually any gene during embryogenesis, and facile generation of fluorescently tagged transgenic animals [14, 15]. These advantages have made the zebrafish an excellent model for understanding development, evolution, and diseases such as cancer, aging, anemia, tissue regeneration, and cardiovascular disease [16, 17, 18, 19]. The exquisite transparency and small size of live, embryonic zebrafish make them as accessible to light-based imaging as any other vertebrate model. With age, however, the zebrafish body becomes less transparent, thicker, and increasingly pigmented. By adulthood, pigmented cells cover a majority of the surface of the fish. These characteristics preclude high-resolution light-based imaging. We review the most common and most powerful imaging modalities the research community has used for embryos (Table 2), and the advantages and disadvantages of each method by developmental stage of the zebrafish (Table 3). Our understanding of development and biology will benefit from integration of methods [20]. Despite its clear value after the affected cell types have been identified, electron microscopy will not be discussed as a candidate for high-throughput phenotyping because its small fields of view make it impractical to image whole zebrafish beyond embryonic ages.

Biological function and cellular mechanisms can be revealed by whole-animal morphological phenotyping

To understand why imaging is a key part of phenome projects, it is appropriate to address a fundamental question: “How is imaging related to gene function?” An awareness of the semantics of “function” and of contributory principles from genetics and pathology are necessary to understand the answer. Geneticists interested in gene function frequently study the phenotype of mutants or knock-downs. In this phenotype-driven research, genetic screens for mutant phenotypes are used to identify genes involved in any given function. For example, a genetic screen for morphologically abnormal Drosophila embryos led to the identification of genes that are necessary for embryogenesis, some of which were later found to play a role in human cancer (reviewed by [21]). Zebrafish genetic screens have been pursued for two decades [14], with contributory support from a cross-NIH zebrafish initiative (http://grants.nih.gov/grants/guide/pa-files/PAR-08-138.html).

Whole-organism phenotyping is essential to understanding the biological function of genes. Scientific definitions of gene functions include the molecular/biochemical, cellular, and biological. A specific example illustrates this point. The commonly used “*golden*” zebrafish are homozygous for a nonsense (presumably null) mutation in the potassium-dependent sodium-calcium exchanger, *slc24a5*. At 48 hours of development, they lack pigmentation, while wild-type fish have darkly pigmented cells in the eye and body [22]. Microscopic

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inspection of adult stages shows that the melanin-containing cells, the melanophores, are lighter in color but similar in number to wild-type. Fewer, smaller, and more lightly pigmented melanosomes were shown in *golden* embryos by electron microscopy of skin [23]. In this example, the *molecular/biochemical* gene function identified by cloning was ion exchange. The *cellular* mechanism – control of melanosome morphogenesis – was revealed by microscopic and ultrastructural morphology. However, *only the whole animal, mutant morphological phenotype* identifies the *biological function* of the *golden* gene as pigmentation within pigment cells of the whole animal. Whole-animal morphology of mutants, in cellular detail, is therefore central to understanding gene function.

Whole-animal imaging is necessary for the Zebrafish Phenome Project

Phenotypes caused by single gene deficiencies can cross organ systems in invertebrate and vertebrate model systems, including humans, and can affect different *sets* of organ systems at different developmental times [3, 1, 4]. Some organ systems, especially those having to do with sexual maturity, are most affected at reproductive ages. We therefore need to phenotype animals not only during embryogenesis, when primordial germ cell migration takes place, but also into juvenile stages when sexual differentiation is complete. Phenotyping juveniles and adults is relevant because humans with single gene deficiencies often survive into adolescence and adulthood. It is therefore informative for phenome projects of any model system to include analysis of the whole organism at different developmental ages.

Phenotyping at cell resolution is essential for detecting pathophysiological mechanisms

One hundred and fifty years ago, based on the discovery that all organisms are comprised of cells (the cell theory), Virchow proposed that disease is a cellular process [24••] that can often be characterized by the study of stained tissue sections at cell resolutions (classically, histology). This principle revolutionized medicine and forms the existing gold standard for diagnosis in anatomic pathology. Today, those criteria are supplemented by protein- and gene-specific assays [25].

Imaging whole zebrafish at cell resolutions presents different problems at embryonic vs. postembryonic stages of development (Tables 2, 3). *In vivo, fluorescence-based* imaging during days 1 and 2 of zebrafish development takes advantage of embryonic transparency. Imaging fluorescently-tagged proteins by laser confocal, two-photon, and sheet microscopy [reviewed by 13] has allowed the monitoring of biological processes such as cell movements and cell division in real time [26, 27•,28•, 29•, 30]. Some exciting, high-throughput methods involving microfluidic handling of embryos have been developed [31, 32•, 33]. But how might pigmented and larger fish be best imaged at cell resolutions? Block-face reconstructions have yielded exciting results for the whole human [34]. A derivative of that method based on fluorescence imaging of cut surfaces, episcopic fluorescence image capture (EFIC) has great promise, but has not yet proven capable of achieving resolutions at or below 1 μm, and is unlikely to approach the throughput needed for the zebrafish phenome project due to built-in limitation of having to alternate between cutting and imaging the entire surface for each of potentially thousands of sections through whole fish [35, 36, 37]. MRI has been used for other model systems [38, 39], can be used to image whole zebrafish through adulthood, provides unique contrast, can be used as a molecular probe, and can serve as a means of high-throughput phenotyping and as a reference scaffold for other imaging methods [40]. It is presently impractical to generate useful voxel resolutions of less than ~8 μm using MRI, which precludes the detection of small cell types. Optical projected tomography (OPT) was used to rapidly generate a labeled on-line atlas through the life-span

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of the zebrafish [41•, 42, 43], but does not reach cell resolution (Tables 2, 3). Optoacoustic imaging has the ability to derive functional information such as oxygen tension [44, 45•], but also cannot provide cell resolution through the full volume of an entire organism. In sum, a variety of *in vivo* imaging techniques have different advantages, but those that produce images of cell resolution have fields of view too small to cover the entire fish older than about 2 days of development (Table 2). We are left with only two methods to image whole, pigmented larval, juvenile and adult zebrafish at cell resolutions: 1) histology, which can yield 2D sections of ~0.25 μm in-plane pixel resolution, and 2) microCT, which yields isotropic, 3-dimensional volumes of ~1 μm voxel resolutions.

Histology

Histology is a standard and detailed way to relate mutant phenotypes to human disease. The principles of cellular pathology – based on the cell theory – serve as a foundation for modern medicine and tell us that disease processes are revealed by the study of tissues at cell resolutions [24, 46••]. Tissue sections allow us to identify a majority of cells, to determine whether and how cells and tissue may be abnormal, and to detect unusual cell types, foreign organisms, and/or unusual deposits or foreign matter. For example, the different possible causes of a human lung mass – types of cancer, a benign tumor, acute infection, chronic infection with a fungus, or foreign body reaction – can be easily distinguished by histology with no more than a standard hematoxylin and eosin stain. These distinctions are difficult to impossible without cell resolutions (pixels of about 1 μm^2 or less), and motivate pathologist-driven quantitative histology for the mouse phenome project [47••].

Array technology facilitates histology for small fish [48•, 49•, 50], has been used for genetic screens [2], and greatly facilitated the creation of an online atlas by the Cheng lab's atlas team at Penn State (www.zfatlas.psu.edu). Whole adults fit into a single cassette. Histology is a standard tool not only for human diagnosis [46••], but also for toxicity testing [51] and for the characterization of mouse mutants [52], facilitating comparisons between normal and diseased zebrafish [53], mice, and humans. These considerations make histology an essential tool for phenotypic screens, especially in support of translational research. The future development of automated and quantitative analysis of histological data may address the common absence of morphological data from systems biology analyses [54].

MicroCT

MicroCT is a tomographic technique by which a series of hundreds to over a thousand x-ray projection images are taken at each of multiple angles over at least 180° through a fixed, intact specimen, from which a 3-dimensional volume is computationally reconstructed [55•]. Contrast within the reconstructed volume is proportional to the attenuation of the X-rays that pass through the sample, and can be scaled by modulating the energy of the X-rays (measured in keV) and by altering the absorption of the sample using heavy metal stains (high atomic number, Z). MicroCT specimens can be left intact for reimaging as technology improves.

There are two types of microCT imaging relevant to the current discussion: commercial microCT and synchrotron-based microCT [Fig 1, reviewed by 56••]. Commercial scanners use a cone-beam imaging geometry in which polychromatic, low-flux X-rays emanate from a focal spot, whose size ("spot size") is a tradeoff between resolution and X-ray flux. The so-called Feldkamp reconstruction algorithm converts a series of projection images generated by the scanner into stacks of aligned, digital, 2D slices. Isotropy of the voxels (cubic, rather than elongated rectangular shape) allows the digital volume to be viewed and re-sectioned in any orientation (e.g. coronal, sagittal, transverse, oblique) without loss of

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resolution. The size of the field of view is inversely proportional to resolution since any given length is distributed across the same number of pixels, so optimizing the balance between these factors is one of the challenges of this field. Cone beam microCT imaging achieves cell resolutions only for the smallest of animals, and at scanning speeds that extend over multiple hours. Only a portion of the X-ray spectrum in commercial X-ray tubes is of sufficient energy to traverse a larger specimen, contributing to artifact and longer scan times. Cone-beam microCT has been proposed as a potential high-throughput screening tool for phenotyping mouse embryos [57•], but neither the faster 27 μm voxel size scans, nor the alternative 8 μm voxel scans with a 6-fold slower scanning time will allow scoring of individual cell types. Even if commercial microCT reach cell resolutions for whole animals, their low X-ray flux is a barrier to throughput and their X-ray polychromicity precludes the use of phase contrast.

Synchrotron microCT uses parallel-beam geometry and monochromatic, highly coherent X-rays [58•] (Figure 1) whose energy can be customized to the size and composition of the subject. The coherence can be used for phase-contrast based edge enhancement, which is modulated by changing the distance between sample and sensor (Fig. 1) [56••, 58•]. Low-energy (“soft”) X-rays in the <1keV “water window” corresponding to the K shell absorption edges of carbon (284 eV) and oxygen (543 eV) can be used to image organelles within single cells within fields of view in the range of 15 μm [59•]. Higher-energy (“hard”) x-rays of 10 to 25 keV are needed to penetrate samples as large as zebrafish stained with a variety of heavy atoms whose absorption maxima lie within that higher range. The latter technology is available at third-generation synchrotrons such as the Advanced Photon Source at the U.S. Department of Energy’s Argonne National Labs in Argonne, IL, the European Synchrotron Radiation Facility in Grenoble, France, and Japan’s Spring-8. Their high flux (equivalent to brighter light in photography) allows for shorter exposure times, and therefore higher throughput. Unstained samples are scanned to image bone, as seen for a commercial scan of a zebrafish juvenile (Fig. 2). High-quality imaging of soft tissues requires the use of contrast agents containing heavy metals such as osmium, uranium, iodine, and tungsten [56, 60, 61•, 62••]. Increases in quantum efficiency and size of both scintillator and CCD chips, together with modifications to X-ray and light optics will bring us closer to the resolution, field of view, and speed needed for high-throughput imaging of zebrafish for the Zebrafish Phenome Project. Comparing results between microCT and histology will provide critical validation for each methodology, based on the 3D information content of microCT and the power of interspecies cross-referencing and availability of special stains in histology. Since microCT images have high resolution over the entire embryo, they may be a good scaffold with which to integrate data that lack anatomical context, or that have small fields of view.

The zebrafish is the only well-developed vertebrate genetic model that is small enough to image the whole animal at cell resolutions using microCT. For existing chip dimensions of 2048 × 2048 pixels, assuming that animals can be scanned one section at a time at full width, resolution is limited by fields of view within which the width of the animal can fit (Table 4). The fields of view for synchrotron microCT at beamline 2-BM at Argonne National Laboratory are about 2.9^2 and 1.5^2 mm for 5x and 10x optics, respectively. Distributing 2048 pixels across those fields of view yields calculated voxel sizes on the order of about 0.75^3 and 1.4^3 μm for scans of larvae and juvenile zebrafish, respectively. This compares with pixel sizes of about 0.5^2, 1^2, and 2^2 μm associated with 20x, 10x and 5x magnification for scans of histological sections that were originally created at 0.25^2 μm pixel resolution with the model XT commercial scanner from Aperio technologies (Vista, CA; Cheng lab, unpublished). Presently, even the larger field of view at 2-BM is too small for mature mouse embryos of 8mm diameter.

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Challenges and Solutions

Image data from live and fixed fish need to be made available to the zebrafish community in a way that is integrated between morphological, behavioral and physiological assays and across model systems, including humans. Meeting this challenge will require the use of anatomic and phenotypic ontologies that cross model systems [9, 26, 42, 63], integration of data across imaging modalities [64], and integrating across multiple imaging modes, including those, such as electron microscopy, that will be used for more detailed study of detected cellular phenotypes [65]. Among the greatest deficiencies in phenomics today are 1) a shortage of annotators who have a wide range of knowledge of human and animal disease [66], 2) a high frequency of incorrect and inadequate phenotyping [8], and 3) a lack of a comprehensive set of tools for generating quantitative data associated with morphological abnormalities.

How critical is the challenge of throughput for the phenome project? Consider for the moment 10,000 mutant lines. Several ages need to be imaged per mutant, and at least 3 experimental and 3 control individuals imaged per age. For each animal tested, 3D reconstructions include thousands of slices through the animal in each orthogonal direction (transverse, coronal, and sagittal). A minimum analysis of just 15 images along one orientation yields 2,700,000 images (10,000 genes × 3 ages × 6 individuals per age × 15 slides/individual) for interpretation. Sample processing, sample loading, imaging, and computational reconstruction would have to keep up, and the rate of image analysis would be 2,700,000/(50 weeks/year × 5 days/week × 5 years) = 2,160 images per day. Even semi-quantitative assessment of morphological features used in pathology requires training, experience and reference materials. Pathologists are insufficient in number to interpret so many images, and variation in training, fatigue, and experience would be expected to introduce human error.

New computational methods for quantitative image analysis for both 2D histology and 3D analysis can ameliorate the problems of personnel and expertise. The translational relevance of interpretation is dependent upon medical and veterinary expertise. Individuals with that expertise know what types of patterns are important to use as diagnostic criteria, and those with some experience with programming or working with programmers know how to suggest realistic algorithms for software solutions. Likewise, programmers who can understand biological and pathologic vocabulary will more likely create usable solutions and make fewer errors caused by misunderstanding. Our experience in interdisciplinary environments supports the idea that individuals with cross-training in some combination of anatomy, pathology, model system genetics, programming, engineering and bioinformatics would likely be able to design and write high-throughput segmentation, pattern-recognition, morphometric, and analytical software with user-friendly interfaces for data mining and web-based community input (“crowdsourcing”). There is potential synergy between the computational approaches developed for histology, microCT, and live-animal imaging (Figure 3). We anticipate unique contributions to systems biology and that the derived tools will be usable across multiple model systems and fields of image-based inquiry.

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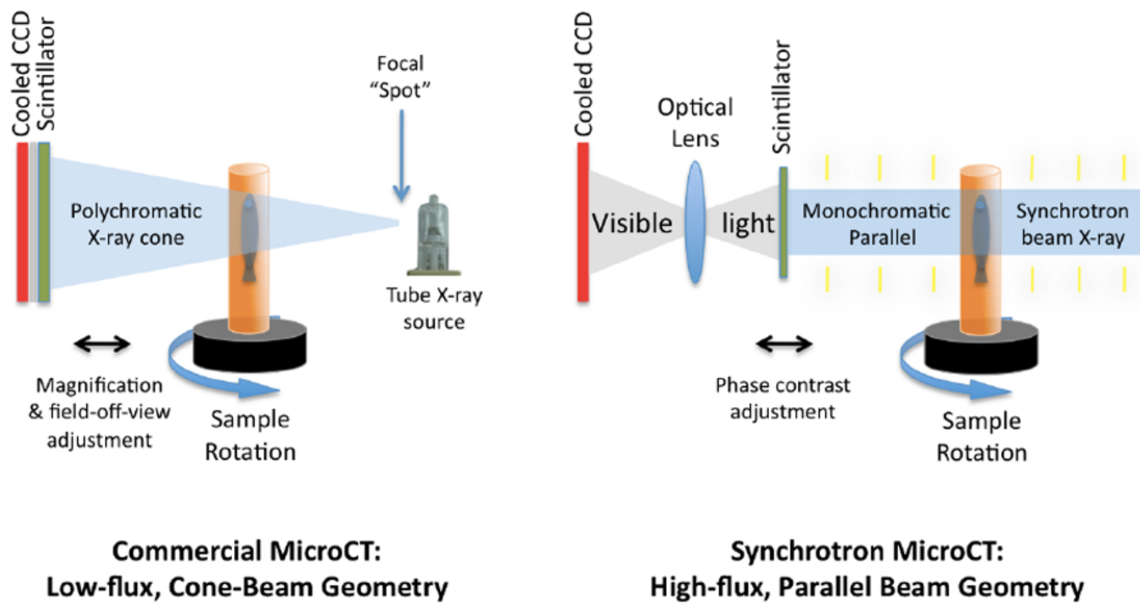

Figure 1. Comparison of Synchrotron Parallel Beam vs. Commercial Cone Beam MicroCT geometry

In cone beam microCT, the X-ray comes from a focal spot, for which diameter is inversely proportional to resolution and directly proportional to flux. The flux (brightness) is low. The cone shape of the beam allows magnification of the desired area by adjusting the relative position of the sample and scintillator to the focal spot, and can be used to focus a subarea of the specimen across the full area of the scintillator. These scintillators may be coupled by fiber optics to the cooled CCD. In synchrotron microCT, X-rays are of parallel geometry, monochromatic, phased, and of high flux. There is no geometric specimen magnification by the X-ray. Edge enhancement by phase contrast is made possible by phased monochromatic X-ray, and is adjusted by changing the sample-to-scintillator distance. The transmitted X-ray induces light in the scintillator, which is projected through optical lenses (e.g. 5x or 10x microscope objectives) whose magnification onto the cooled CCD determines optical magnification of the scintillator surface. For both types of microCT, the specimen is rotated over at least 180 degrees, commonly with one projection image taken every 0.1 to 0.3 degrees of rotation (yielding 1800 and 600 images, respectively).

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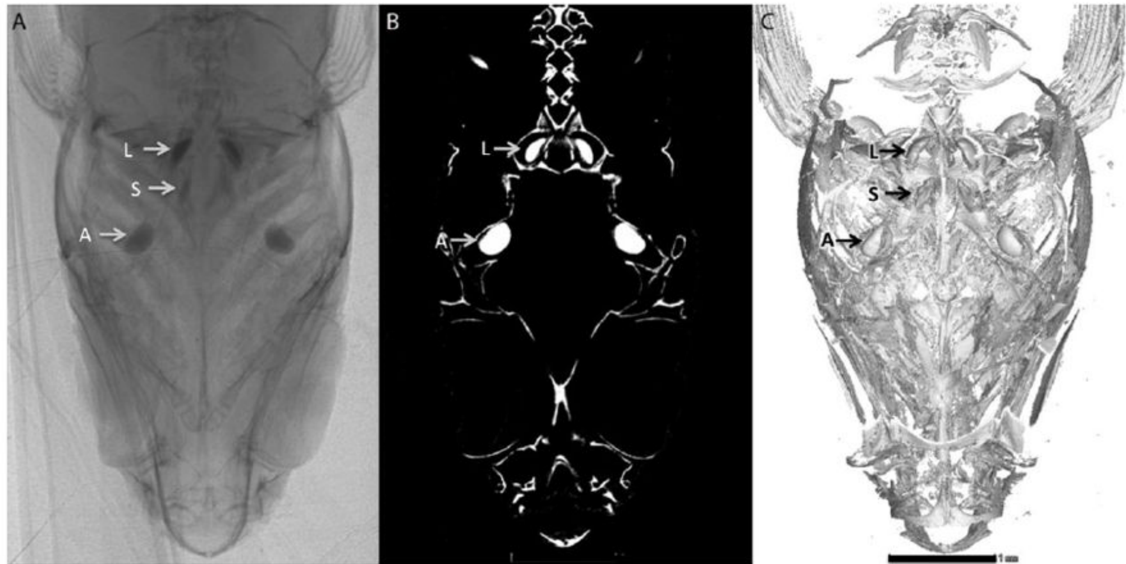

Figure 2. Micron-Scale Computed Tomography (microCT)

A series of processing steps allows 3D models to be generated from microCT data. Demonstrated here is the application of these steps to reconstruct a zebrafish's skull and inner ear. (A) One of 600 x-ray projection images taken, over ~4 hours, through 180° of the head of an unstained, 60 dpf juvenile zebrafish wrapped in parafilm (one image every 0.3°). (B) One of 1500 digital, coronal 2D slices generated by applying the Feldkamp cone-beam reconstruction algorithm to these 600 images. (C) A screen-capture of part of a 3D model generated from these 1500 2D slices in a Volume Graphics software package known as VGStudio Max (Heidelberg, Germany). The arrows labeled L, S, and A, are the lapillus, sagitta, and asteriscus, which are found in the utricl, saccule, and lagena, respectively [67]. A similar comparison can be accessed at http://www.zfatlas.psu.edu/comparison.php?s[]=262&s[]=267&s[]=268. Scale bar, 1mm.

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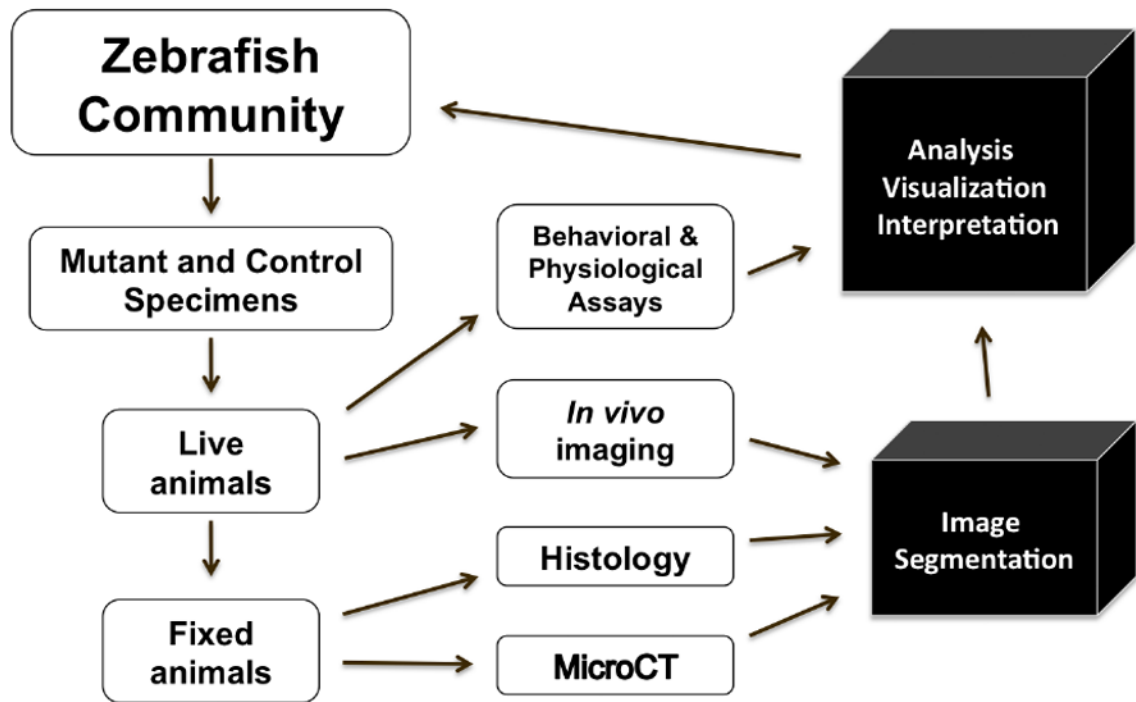

Figure 3. Zebrafish Phenome Project Paradigm

For the phenome project, mutant and control animals will be generated for live and fixed animal studies. The imaging data will benefit from segmentation software, as well as software to facilitate analysis, visualization, and integrated interpretation (black boxes). The output, as well as raw data, would then become available to the zebrafish and other research communities.

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Table 1

Ideal Features of Imaging for the Zebrafish Phenome Project

- Whole animal
- Cell resolutions (~ 1 μm)
- Embryos, juveniles and adults
- Isotropic (each voxel dimension same)
- Digital output
- High-throughput
- Quantitative Digital analysis
- Fluorescence *in vivo* when possible
- 3D modeling

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Table 2

Technical features of imaging modalities being considered for the Zebrafish Phenome Project.

Technology	Resolution Limit	Maximum Sample Diameter	Radiation	References	Advantages	Disadvantages
Histology (2D)	0.22 μm (determined by lens numerical aperture)	Usually ~0.9 × 5cm; available to	Visible Light	[49,53]; www.zfatlas.psu.edu	Standard translational tool, high 2D resolution, whole animal, online atlas, laser capture microdissection possible	2D, destructive, step sections cover most of fish, but leaves most unseen
Confocal Microscopy (Scanning Laser)	~0.4 μm	~determined by N.A. of objective	Visible Light (laser spot)	[26, 27]	Resolution, gene specificity, elaborate 4D software developed	Limited penetration, small field of view, nonisotropic, slow, phototoxic
Selective Plane Illumination Microscopy (SPIM)	5-10 μm	1-5 mm	Visible Light (laser, or laser sheet)	[27,28]	Whole, live animals imaged quickly(SPIM; 6 sec/frame),up to ~500 μm depth, 6 μm resolution, gene specificity by fluorescence	Poor resolution for thicker specimens, not for pigmented specimens, nonisotropic, limited field of view
Optical Projection Tomography (OPT)	11-26 μm	1-10 mm	Visible Light	[41,43], http://www.fishnet.org.au/	Whole, live animals imaged quickly, gene specificity with transgenics, online atlas	Poor resolution for thicker specimens, not for pigmented specimens, not cellular resolution, nonisotropic
Block Face Imaging (e.g. episcopic fluorescence image capture - EFIC)	Currently > 2 μm for whole animals	Large	Visible Light	[35-37]	Little distortion, resolution set by section thickness and surface imaging optics; used for human embryos and adults	Destructive; slowed by cycles of sectioning and imaging; generally nonisotropic; resolutions for whole vertebrate embryos not yet < 2 μm
Magnetic Resonance Microscopy	19.5 μm	mm to cm	Magnetic Field	[38-40]	Excellent contrast, tissue resolution, isotropic, nondestructive	Low-throughput; not cellular resolution, few high field machines
Functional Optoacoustic Microscopy	~6 μm	~3 mm (deeper-> lower resolution)	laser light and ultrasound	[44,45]	Live animal in situ imaging, functional (e.g. oxygen saturation), fast	Poor resolution with thicker specimens nonisotropoic.

Technology	Resolution Limit	Maximum Sample Diameter	Radiation	References	Advantages	Disadvantages
MicroCT (low-flux, parallel-beam, e.g. Lawrence Berkeley National Laboratory)	10-15 nm	<40 nm	Soft X-ray	[59]	No special stains necessary, organellar resolution, isotropic, nondestructive	Small field of view; cryoimaging is difficult for objects of zebrafish embryo size; not usable for whole animals.
MicroCT (low flux, cone-beam, commercial)	~1.1 µm (nominal, limited by spot size)	1.15 mm	tube X-Ray	[57-61-62], http://www.xradia.com, http://www.skyscan.be/home.htm	Whole animal, adjustable field of view, isotropic, nondestructive	Fixed only, long scan times; only largest cells visualized
MicroCT (hi-flux, parallel-beam, third generation synchrotron)	~1 µm	1.5 / 2.9 mm (limited by scintillator resolution and CCD chip size)	synchrotron hard X-Ray	[56,58,60], http://www.aps.anl.gov/Xray_Science_Division/Xray_Microscopy_and_Imaging/	Whole-animal, fast imaging, cellular resolution, isotropic, nondestructive	Fixed only, few facilities (will require national resource for high-throughput)
NanoCT (low flux, cone-beam, commercial)	80-150 nm	~11 mm	tube X-Ray	http://www.skyscan.be/home.htm	high resolution, available to single lab, isotropic, nondestructive	Fixed only, Small field of view, slow imaging
NanoCT (hi-flux, parallel-beam, third generation synchrotron)	30 nm	~1 mm	synchrotron hard X-Ray	http://nano.anl.gov/research/nanoprobe.html	High resolution, fast, isotropic	Fixed only, small field of view, will require national resource for high-throughput, sample may be damaged by huge flux

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Table 3

Suitability of imaging modalities for imaging zebrafish at different developmental stages for the Zebrafish Phenome Project.

Technology	Cell Resolution (~1 µm pixel or voxel dimension)	Throughput Potential for ZF Phenome Project (<1 minute/ animal)	Whole Unpigmented embryos	Whole Pigmented Larvae (~1mm width) @ Cell Resolution	Whole Pigmented Juveniles (~3mm width @ Cell Resolution	Whole Pigmented Adults (~7mm width @ Cell Resolution	Isotropic Voxels	Comparable Human and Mouse Data
Histology (2D)	Yes	Yes	Yes	Yes (2D)	Yes (2D)	Yes(2D)	No	Yes
Confocal Microscopy (Scanning Laser)	Yes	No	Yes	No	No	No	No	No
Selective Plane Illumination Microscopy (SPIM)	Almost	Yes	Yes	No	No	No	No	No
Optical Projection Tomography (OPT)	No	Yes	Yes	No	No	No	No	No
Block-face reconstruction (e.g. episcopiec fluorescence image capture - EFIC)	Not yet	Unlikely	Yes	Yes	Yes	Yes	No	Yes
Magnetic Resonance Microscopy	No	No	Yes	No	No	No	Possible	Yes
Functional Optoacoustic Microscopy	No	Yes	No	No	No	No	No	Yes
MicroCT (low-flux, parallel-beam, Lawrence Berkeley National Laboratory)	Yes	No	No	No	No	No	Yes	Not yet
MicroCT (low flux, cone-beam, commercial)	Almost	No	Yes	Not yet	No	No	Yes	Yes
MicroCT (hi-flux, parallel-beam, Third-Generation Synchtrotrons)	Yes	Yes	Yes	Yes (currently in segments)	Yes (currently in segments)	Will require larger scintillator and CCD chips	Yes	Yes (indirect, through histology)
NanoCT (low flux, cone-beam, commercial)	Subcellular	No	No	No	No	No	Yes	Not yet
NanoCT (hi-flux, parallel-beam, Argonne National Labs)	Subcellular	No	No	No	No	No	Yes	Not yet

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Table 4

Comparison of model system sizes.

Structure	Greatest width (approximate)					
	Human Adult	**Mouse Adult**	**Mouse Embryo**	**Zebrafish Larva**	**Zebrafish Juvenile**	**Zebrafish Adult**
Body length	180 cm	7 cm	15 mm	4 mm	14 mm	3 cm
Body scanning width	75 cm	3.5 cm	10 mm	*0.55 mm	*2.6 mm	5.5 mm
Brain scanning width	24 cm	15 mm	5.6 mm	1 mm	2 mm	2 mm
Eye	25 mm	3.4 mm	1.2 mm	0.25 m	1.1 mm	2 mm
Red blood cell	8 µm	6.8 µm	6.8 µm	5 µm	5 µm	5 µm