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Genes into geometry: imaging for mouse development in 3D

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Mammalian development is a sophisticated program coordinated by a complex set of genetic and physiological factors. Alterations in anatomy or morphology provide intrinsic measures of progress in or deviations from this program. Emerging three-dimensional imaging methods now allow for more sophisticated morphological assessment than ever before, enabling comprehensive phenotyping, visualization of anatomical context and patterns, automated and quantitative morphological analysis, as well as improved understanding of the developmental time course. Furthermore, these imaging tools are becoming increasingly available and will consequently play a prominent role in elucidating the factors that direct and influence mammalian development.

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Introduction

The development of an organism is a complex process that proceeds with remarkable and fascinating precision. Organs, limbs, bones, and other structures are routinely generated in the right location with appropriate size and shape. Various tissues and systems such as arteries and veins, alveoli and vasculature, and limbs and nerves are dependent on one another for proper function and develop in a coordinated and concurrent fashion. While much of this developmental program appears to be under tight genetic control, there is also significant stochastic patterning, functional tuning and tissue remodeling. Elucidation of the role of each of these mechanisms in the intricate developmental process is one of the central objectives of developmental biology.

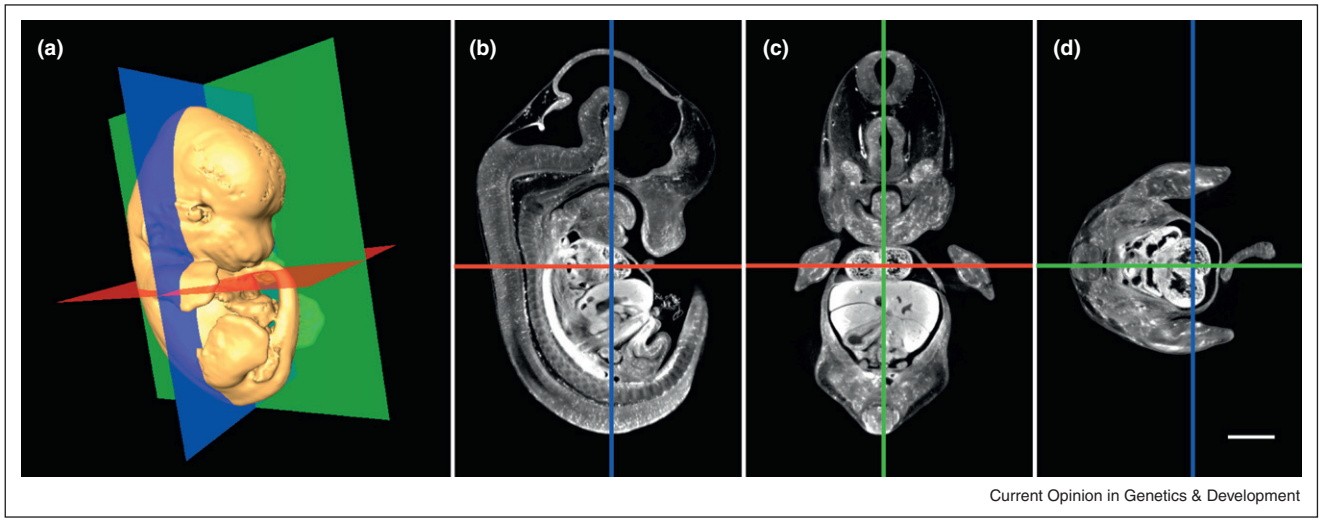
Morphometry—size, shape and form—of anatomical features is frequently used to characterize normal and abnormal development. Excellent descriptions of high-level patterning processes have already been generated on this basis. However, these descriptions frequently lack quantification of the morphological changes and a detailed understanding of the underlying physical mechanisms. As growth and development inherently occurs in extended three-dimensional (3D) space, 3D data is necessary for improved characterization of development. At very early stages and in small species such as the zebrafish or drosophila, it is sometimes possible to visualize morphometry with modern confocal or two-photon microscopy *in situ* and even *in vivo*. However, in the mouse, such visualization is not possible at any but the earliest of developmental stages. Moreover, morphometric analysis does not generally require the subcellular resolution of microscopy, rather an extended field-of-view and 3D context. Consequently, imaging tools that operate at the mesoscopic scale (2–50 μm) with isotropic resolution over a 10–50 mm field-of-view (FOV) are most appropriate for effective morphometric analyses in the developing and adult mouse.

A combination of new imaging tools, including some adapted from clinical radiology, now allow for 3D imaging of the mouse at the mesoscopic scale. Magnetic resonance imaging (MRI), micro-computed tomography (micro-CT), and ultrasound biomicroscopy (UBM) can all provide digital morphometric characterizations. Optical methods, such as optical projection tomography (OPT), and fluorescence light-sheet microscopy, and episcopic 3D imaging techniques, such as episcopic fluorescence image capture (EFIC) and high-resolution episcopic microscopy (HREM), can generate this morphometric information at even finer resolution but over a smaller FOV. In this review, we describe the current potential of these 3D imaging methods in the study of morphometry, patterns and phenotyping. In particular, we present a variety of examples of how 3D imaging provides comprehensive coverage for phenotypic screening, enables visualization of anatomical context and patterns, allows for automated and quantitative analyses, and assists in understanding the developmental time course. These benefits confer improved efficiency, sensitivity and quantitation in developmental studies, and will prove invaluable in the international effort to phenotype a knockout mouse for every gene in the genome [1••].

Comprehensive phenotypic screening

Three-dimensional imaging provides complete coverage of the mouse so that phenotypes in multiple organs and

Figure 1

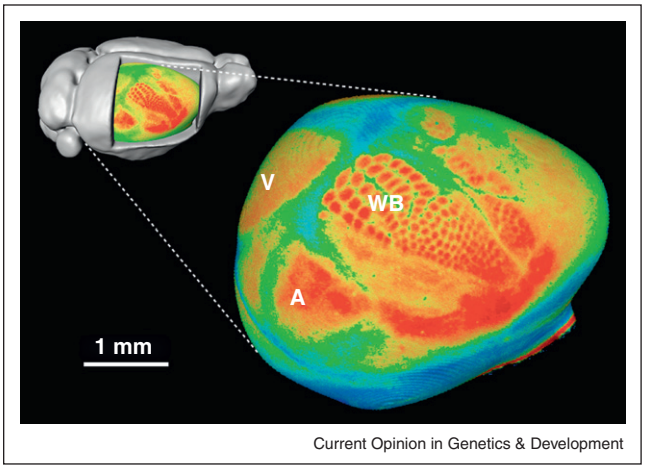


3D whole volume image of an E12.5 mouse embryo generated with OPT. The surface rendering of the whole-embryo volume **(a)** illustrates the 3D nature of the data set, while highlighting the context of the sagittal **(b, green plane)**, frontal **(c, blue plane)**, and transverse cross-sections **(c, red plane)** in the other panels. Scale bar is 2 mm. Whole volume coverage and the ability to section the data set at any orientation are two main benefits from 3D imaging.

structures can be assessed simultaneously. This is equally important in screening applications, where altered morphometry may appear in any location, and in targeted mutations of previously characterized genes, as phenotypes frequently appear in unanticipated structures or regions. Optical imaging methods, including OPT [2], EFIC [3], HREM [4] and fluorescence light-sheet microscopy [5,6•], are best-suited for imaging of the early to mid-stage embryo (Figure 1). Tissue autofluorescence provides excellent contrast for morphometric analyses; on this basis, whole-embryo 3D images or atlases have been reported for OPT [7] and HREM [8], starting at ages as early as E6. MRI [9,10•,11••] and CT [12–14] are alternatives well-suited to imaging late-stage embryos, neonates and adults.

Detection of developmental abnormalities has been reported most frequently in the brain and heart, although this reflects more the prevailing interests in the developmental biology community than any methodological limitations. Within these structures, 3D imaging can detect phenotypes that are diffuse or focal in nature. For example, multiple volume deficits are observed using MRI in developing mice exposed to alcohol *in utero* [15] and in mice lacking important growth factor receptors [16]. More focal abnormalities are detected after a mutation of genes affecting development of the cerebellar vermis [17], cerebellar nuclei [18••] or commissural tracts [19,20••]. In the heart, abnormalities in chamber volume, septation or outflow tract connection can be readily appreciated from 3D data [21]. With the extended coverage of whole-embryo imaging, phenotyping of cardiac septa, outflow tracts and surrounding thoracic structures including the thymus can be accomplished simultaneously [22–24]. Three-dimensional optical imaging has also revealed morphological phenotypes in a number of other locations including the liver [25], lungs [26] and kidneys [27•].

Figure 2



Visualization of layer IV of the somatosensory cortex with OPT. The surface defined by cortical layer IV was extracted from a 3D OPT autofluorescence image (bottom right). Intensities from the OPT volume were mapped onto the surface to show cortical structure in its native geometry. The rendering can be shown within the context of the whole brain (top left). Visual (V), auditory (A), and whisker barrel (WB) sensory regions are well defined, but are extremely difficult to visualize and characterize using 2D imaging methods.

Anatomy and pattern visualization

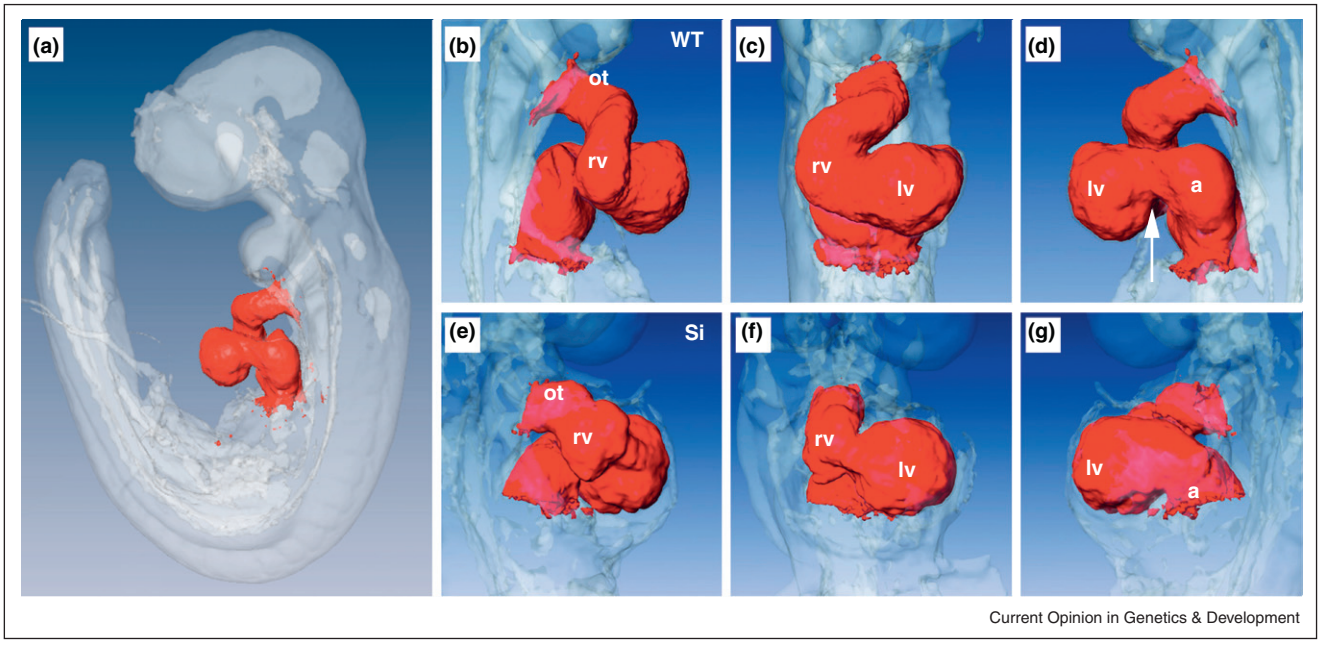
Many anatomical features are simply not amenable to two-dimensional visualization. This is true at all stages of development and even for many structures with relatively simple geometry. An example of the latter includes layer IV of the mouse somatosensory cortex, which lies on a smoothly curved surface that can be elegantly visualized in its natural geometry with OPT (Figure 2). More complex geometries are impossible to visualize in anything but 3D space. Facial development, in which the relatively simple branchial arches develop into the various structures of the face, throat and neck, requires 3D interpretation as reported with micro-CT [28–30] and with OPT [31]. Patterning of the limb is similarly complex and requires the coordinated development of tendons, muscles, bones and vasculature and is well shown with OPT [32–34].

Heart development is an intricate process that transforms a linear heart tube through looping, chamber formation, septation and valve formation steps within a network of intertwining inflow and outflow tracts. Three-dimensional imaging is critical for proper visualization of the heart geometry at each of these steps, and some phenotypes are very likely to be missed without it [35]. OPT of *Smarcd3* deficient embryos with multiple fluorophores, for example, reveals an altered outflow tract, hypoplastic right ventricle and missing atrio-ventricular canal compared to wild-type embryos [36••] (Figure 3). Other cardiac phenotypes may range from a complete failure to form the heart chambers to minor septal or valve defects [37–39].

Vascular networks present perhaps the clearest example of visualizations that can be performed only in 3D. MRI and CT have been the standard for vascular imaging over large FOVs for sometime. Perfusion of a vascular contrast agent enables vascular phenotyping in the brain [40], kidney [41], placenta [42,43] and lung [44•] (Figure 4). OPT of the mouse vasculature provides sufficient resolution for visualization of the most dynamic stages of vascular development, between E8.0 and E10.0 in the mouse embryo, showing progression from isolated vascular cells to a complex vascular plexus with subsequent vessel pruning [45••,46]. Other structures with branching architectures, such as the uretic tree of the kidney [27•], necessitate similar 3D visualizations.

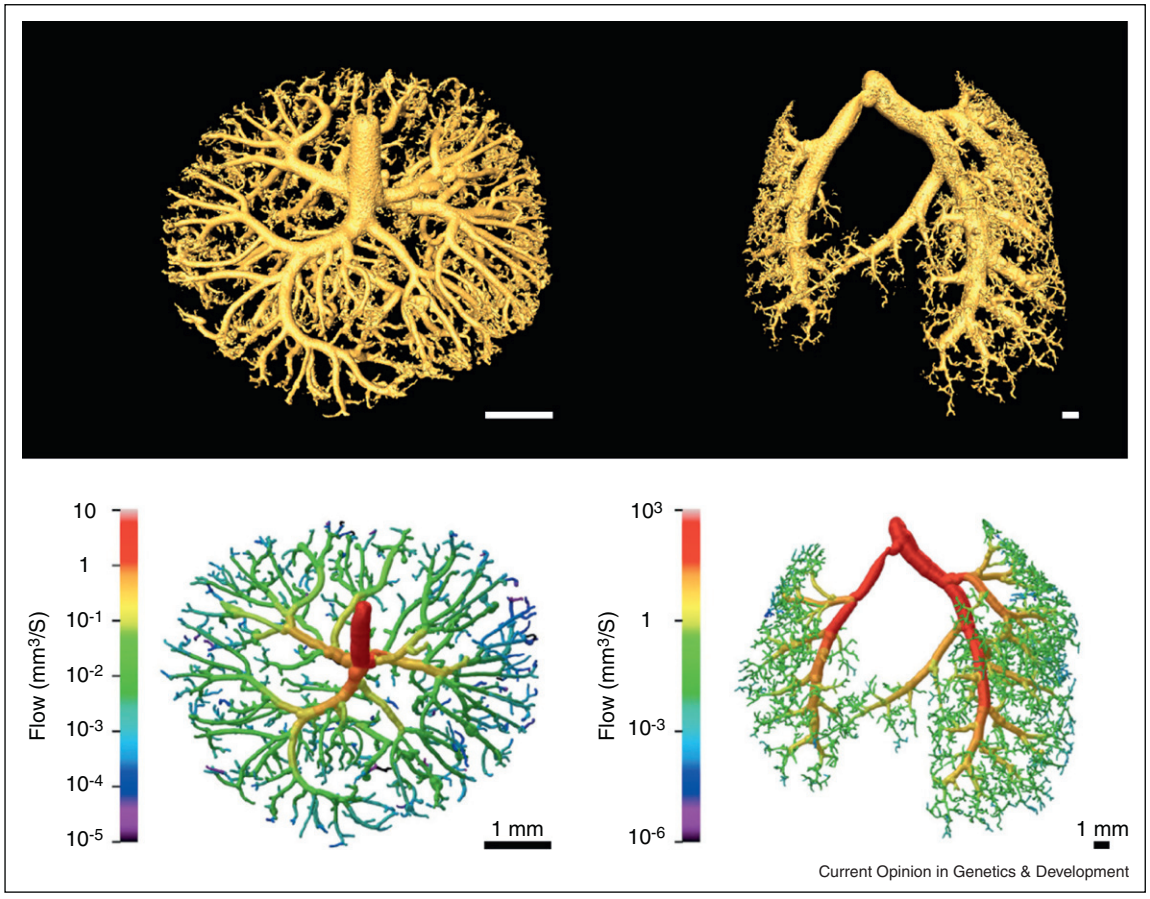
Optical imaging provides the additional capability to map gene expression patterns within a 3D anatomical context. This can be achieved either by staining with fluorescent antibodies, or detection of transgenicly expressed fluorescent proteins. On the one hand, this provides a comprehensive map of gene expression through the entire body for identifying the diverse roles

Figure 3



Rendered OPT images showing cardiac phenotypes in *Smarcd3* knockdown (Si) embryos at E9.5 (compared to control, WT embryos). The heart is colored red and the rest of the embryo is translucent. The whole-embryo image **(a)** shows the context from which the anatomy in panels **(b–g)** are displayed. The *Smarcd3* knockdown (Si) shows a shortened outflow tract (ot), a hypoplastic right ventricle (rv) and atrium (a), and a missing atrio-ventricular canal (arrow). Each of these phenotypes is most easily appreciated from a different orientation, including views from the right **(b and e)**, front **(c and f)** and left **(d and g)**. The combination of these phenotypes would be difficult to characterize using a single stack of 2D images.Figure is adapted from [36••].

Figure 4



Visualization of vascular patterning with micro-CT imaging after perfusion of a radio-opaque contrast agent. Examples of the placenta (left) and lung (right) are provided. The 3D data allows for visualization of the tree, as for instance with an iso-intensity surface rendering (top), and semi-automated segmentation of vessels. Vessel diameters and positions can be extracted from the segmented vascular tree and then used to perform flow simulations and to estimate physiological parameters of interest, including the expected blood flow at each point in the tree (bottom). These geometric and physiological vascular patterning differences can be used to characterize trees from different organs or from different mice. Scale bar is 1 mm. Image reproduced from Yang *et al.*, *Am J Physiol Heart Circ Physiol* 2010 [44•]. Am Physiol Soc, used with permission.

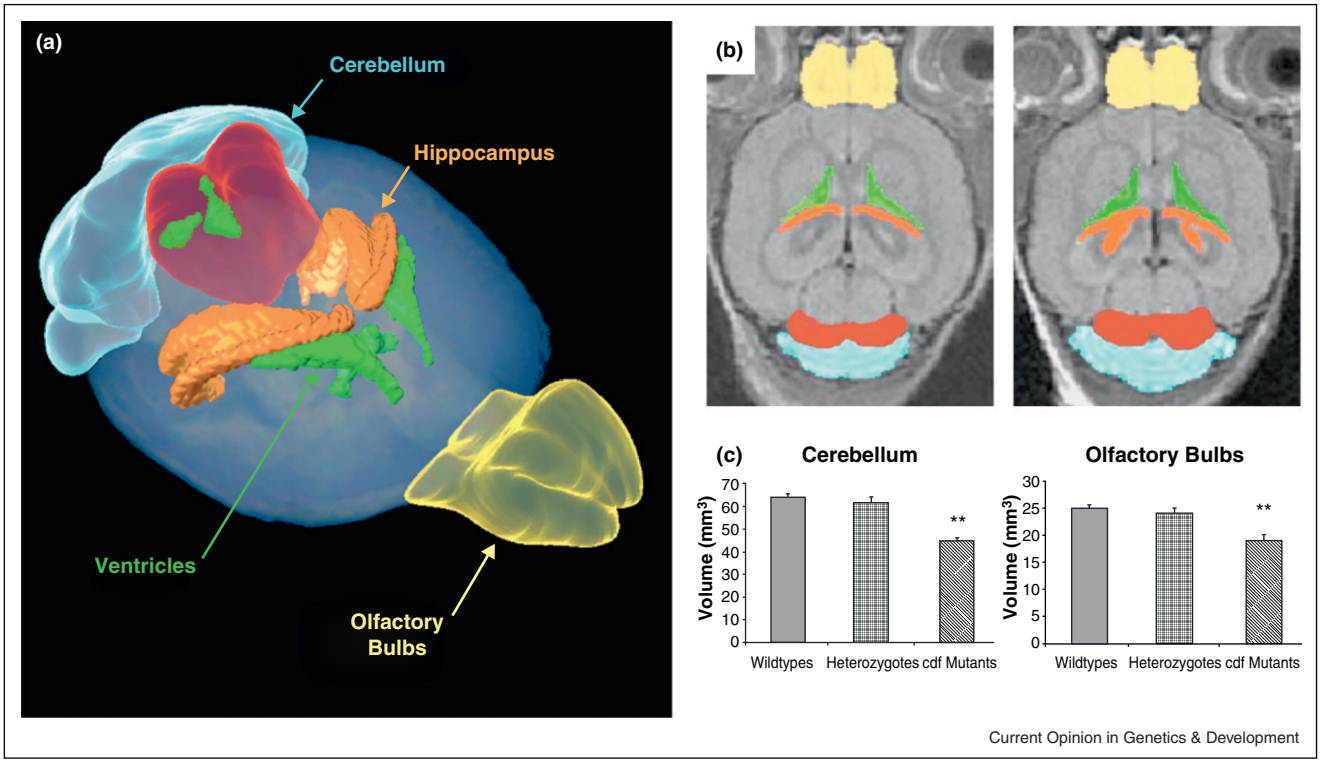
of critical developmental genes [39,47–50]. On the other hand, local mapping of gene expression relative to morphological change, during craniofacial [31] or lung [26] development for instance, provide insight into the possible roles of gene expression in mediating development. Mapping the interplay between genetic expression patterns and morphology is one of the most exciting applications of 3D optical imaging in developmental biology.

Automated and quantitative analysis

A key benefit to the acquisition of 3D image data sets is the capability to perform digital processing for quantification of morphometry. This processing can be performed efficiently with automated methods based on nonlinear registration to identify anatomically equivalent structures, segmented average atlases and voxel-based morphometry [51,52]. Results of this kind have been most frequently reported in the brain [18,53,54]. Volumetric analyses in the cerebral deficient folia mutant (cdf) is an example, where comparison of segmented and annotated structure volumes after MRI revealed an expanded set of morphological phenotypes (Figure 5) [55]. Similar automated computer quantification has revealed abnormalities in structures throughout the brain, including the cortex, hippocampus, cerebellum and other structures [56].

In addition to measuring the volumes of identifiable anatomical structures, localized changes can be highlighted computationally without predefining features of interest. Color maps evaluating volume changes on a pixel-by-pixel basis can be compared to produce 'hot spots' indicative of statistically significant local change. These analyses allow, for instance, quantitative comparison of craniofacial features with micro-CT [57] (Figure 6), and are sufficiently sensitive to detect changes associated

Figure 5



Volume differences between cdf mutant and control brains using MRI and semi-automated volume analysis. Several structures within the mouse brain were segmented from a consensus wild-type average image and rendered in a 3D context **(a)**. The segmented brain regions were mapped to individual mutant, heterozygote and wild-type brains to allow volumetric comparisons as a function of genotype. Individual slices from *in vivo* MRI images are also shown in horizontal cross-section **(b)** where the accuracy of the projected volume segmentations is apparent. The volumes of several brain regions, including the cerebellum and olfactory bulbs **(c)**, were reported as significantly different between the *cdf* mutant and wild-type groups. Figure reproduced from [55] with permission from the Society for Neuroscience.

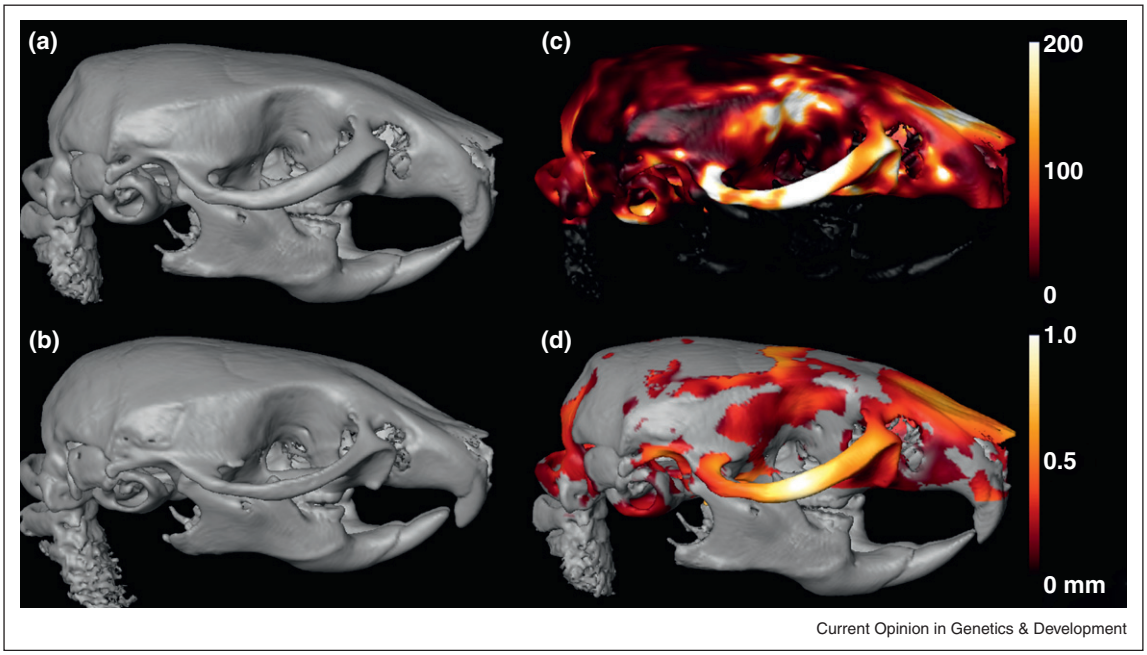
with task-related learning in the adult mouse brain [54]. Morphometry appears to be a highly sensitive indicator of underlying dysfunction in the brain; 90% of mice exhibiting behavioral symptoms show detectable neuroanatomical differences as well [58•]. Methods similar to these also apply to phenotyping during embryonic development and allow the identification of anatomical defects and growth retardation [56,59].

Three-dimensional imaging allows the automated measurement of many additional morphological metrics. A map of cortical thickness, for instance, may be of greater interest than cortical volume and finds application in both development and aging research [16,60]. In quantification of vascular trees, where stochastic patterning of small vessels does not result in geometric homology, measurements of vessel diameter, vessel resistance, perfusion volume or flow as a function of position in the vascular tree (Figure 4) may be computed on the basis of 3D images [41,44•]. Such vascular analyses reveal developmental and environmentally induced alterations in placental vasculatures [42,43].

Developmental time series generation

Development, of course, implies a program of change over time. The efficiency of 3D imaging introduces the possibility of evaluating this time course in more detail. Imaging of multiple individual animals each at different time points allows for a high-resolution, representative time series to be constructed based on registration. Several time series of normal development have been generated on this basis. Anatomical MRI time series of fixed specimens have been reported from E6 through E15.5 [9] and from E10.5 through 32 days postnatally [11••] with regular imaging timepoints throughout. A similar developmental time series based on OPT is presented in Figure 7. Vascular-specific developmental time series have also been reported both with MRI [40] and OPT [45••]. In addition, *in vivo* measures of physiological function, such as blood velocity and flow pattern have been recorded with UBM, from E14.5 to adulthood, showing developmental changes in ventricular cardiac function [61]. The ability to visualize morphology, to measure functional parameters, and to compare mutant phenotypes to earlier or later time points in control

Figure 6



Automated computational evaluation of local changes in the skull of a mutant mouse. An average representation of micro-CT images of the skull of wild-type animals **(a)** can be compared to an average of mutant animals **(b)**. A statistical map, the Hotelling T^2, highlights the regions of most significant change in skull structure after accounting for population variability **(c)**. The displacement (in mm) between the wild-type and mutant populations can be mapped in the regions of statistically significant change **(d)** false discovery rate less than 0.05. The example provided is the *Gja1*^{Jrt} mutant, which represents a mouse model of oculodentodigital dysplasia. Identical analyses can be applied with other imaging modalities for automated, volumetric identification of phenotypes.
Image reproduced from Nieman *et al.*, *Physiol Genomics* 2006 [57]. Am Physiol Soc, used with permission.

animals provides the ability to identify abnormalities and quantify developmental delays or accelerations.

Automated and quantitative analyses can further characterize the growth and variability at each time point. This allows comparisons of mutant and normal growth rates, in addition to structural shape and volumes at individual time points. For instance, the growth between individual stages of development can be plotted, to highlight the regions of most active growth [59,62]. This kind of reconstructed temporal analysis compensates for individual growth patterns in mammalian embryos that cannot be imaged three-dimensionally throughout development.

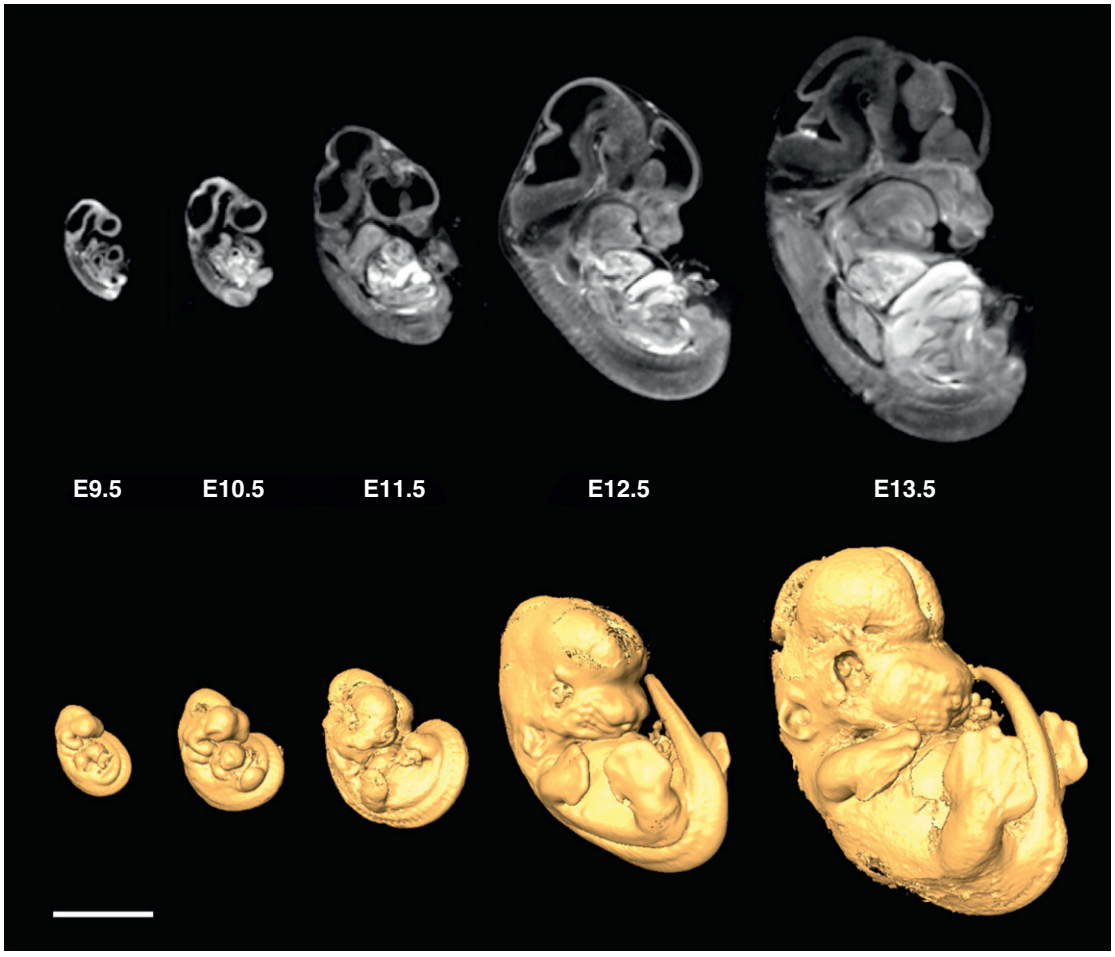
Conclusions

Already, 3D imaging methods are becoming much more widely available. Three-dimensional optical methods are likely to become the most common, due to the fact that they are more easily sited and fit with the workflow in a traditional laboratory setting. However, other imaging techniques, including MRI and CT, will remain important in many applications and represent the only imaging modalities with potential for large FOV *in vivo* measurements. The comprehensive coverage of these 3D methods is crucial for phenotyping.

Image analysis will continue to be an important area of development. The most common automated analyses are likely to become available through manufacturers or directly from academic laboratories, but measurements in a particular application will usually require adaptation. Furthermore, analyses of some structures, notably vasculature, still require the development of new metrics for phenotyping. Incorporation of the temporal component of development into analysis through 3D imaging at several developmental stages will provide the ability to describe when abnormalities first occur and the extent to which development is accelerated or retarded in particular mutants.

In the coming years, 3D imaging and quantitative analysis will become a standard tool in developmental biology for morphogenetic analysis of transgenic and knockout mice. Three-dimensional imaging provides comprehensive coverage for phenotypic screening, enables visualization of anatomical context and complex patterns, allows for automated and quantitative analyses, and assists in understanding the developmental time course. Imaging will play a particularly important role in phenotyping the complete set of single gene knockout animals [1••]. In this fashion, 3D imaging stands to play a central role in

Figure 7



Three-dimensional image time series illustrate the development process. A 4D atlas (3D plus time) can be generated using OPT images of mouse embryonic development from E9.5 to E13.5 at full day intervals. Mid-sagittal sections (top) and iso-intensity surface renderings (bottom) of the mouse embryo at each stage are represented here. Scale bar is 2 mm. Such a time series allows for characterizing organogenesis over normal development as well as identifying irregular morphological maturation in a mutant. Mutant comparisons with earlier and later time points in the series will identify phenotypes causing accelerated or retarded development in individual structures.

dissecting the genetic and physiological factors that control mammalian development.

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- •• of outstanding interest

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