

Electron microscopy in cell biology: integrating structure and function

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Electron microscopy (EM) is at the highest-resolution limit of a spectrum of complementary morphological techniques. When combined with molecular detection methods, EM is the only technique with sufficient resolution to localize proteins to small membrane subdomains in the context of the cell. Recent procedural and technical developments have increasingly improved the power of EM as a cell-biological tool.

With the sequencing of the human genome nearly complete, characterization of the functions of the gene products will be an important next challenge in cell biology. The rapidly growing number of human disorders that are identified as an intracellular membrane-trafficking defect unequivocally shows how protein function is directly linked to intracellular localization and emphasizes the importance of understanding membrane-mediated protein-trafficking pathways. Electron microscopy (EM) is the only technique that can combine sensitive protein-detection methods with detailed information on the substructure of intracellular compartments. This combined resolution power has given EM a central position in defining intracellular protein-distribution patterns and predicts a continuing and increasing demand for EM in cell biology^{1–3}.

Although EM is an established technology, new methodologies have recently been developed to assess the fine-structure identity of intracellular membrane-enclosed compartments. At the same time, the methods to localize molecules of interest to within these compartments have been broadened and perfected. Furthermore, techniques are now evolving that literally add an extra dimension to EM images — electron tomography (ET) for three-dimensional (3D) imaging and CORRELATIVE MICROSCOPY for integrating the imaging of live cells and EM. Here, without the pretension of being complete, we highlight some of the most recent and developing EM techniques, which we believe will have an important role in the future of cell biology.

ImmunoEM: crucial considerations

EM is exploited to its full capacity when its power to visualize membrane compartments

at high resolution is combined with methods that specifically localize the functional components of these membranes — that is, when cellular architecture is integrated with molecular information. For maximal output, we need to strive for the best possible structural preservation and to use the most sensitive protein-detection methods.

Cryofixation. The best available approach so far to immobilize and preserve cellular architecture is probably CRYOFIXATION^{3–5}. HIGH-PRESSURE FREEZING is the only way to freeze biological samples of 10–200- μ m thickness without generating artefacts as a result of ice-crystal formation, and is therefore the method of choice for cells and tissues⁶. In most applications, high-pressure freezing is followed by FREEZE SUBSTITUTION. These preparations are notable because of their sharp membrane delineation and the clear visibility of their cytoskeletal elements. In particular, cellular compartments that are susceptible to shrinkage as a result of chemical fixation — for example, endosomes — retain their turgid shape after high-pressure-freezing fixation (J. L. Murk, M. Kleijmeer and B. Humbel, personal communication). FIGURE 1b shows an example of a Golgi complex that was prepared using this protocol.

When freeze substitution is omitted and frozen cells are viewed directly in the electron microscope, we refer to this method as CRYOEM — a technique that was pioneered by Dubochet and colleagues (for more information, see REF. 4). CryoEM provides the exciting possibility of viewing the cell in what is probably its most natural state. The full procedure, from high-pressure freezing to visualizing slices of frozen material by EM, however, needs further development before it can be

applied routinely^{3,7}. CryoEM of isolated molecules is a rapidly emerging and powerful approach for obtaining structural models of macromolecular complexes in the intermediate resolution range (0.5–2.0 nm). CryoEM can be applied to those complexes that are not suitable for X-ray crystallography because of their size, instability, or the fact that they are available only in insufficient amounts (for a recent overview, see REF. 8). The resolution of cryoEM usually allows the relative positions and orientations of individual components of a macromolecular complex to be determined to within a few angstroms. For example, recently, single-particle cryoEM of the ribosome-bound class I release factor RF2 indicated that binding of RF2 to a stop codon in the decoding centre of the 30S ribosomal subunit induces a conformational change in RF2 that allows it to interact simultaneously with a stop codon and the peptidyl-transferase centre of the 50S ribosomal subunit^{9,10}.

Chemical fixation. Although there are routine protocols for the high-pressure-freezing fixation of biological samples, precise experimental conditions must be established for each sample to prevent the formation of damaging ice crystals, and not all material is suitable for cryofixation. This is why many studies still make use of the more conventional chemical-fixation methods. Chemical fixation, however, induces structural artefacts and might cause the redistribution of particularly small soluble proteins. To weigh up the pros and cons of the two preparation methods for future EM studies, a careful analysis of the possible artefacts that are induced by chemical fixation is much needed. The more laborious high-pressure-freezing procedures might be advantageous for some, but not all, cell-biological questions.

Assessing molecular information. The most widely used approach to detect proteins in cells is IMMUNOEM. Many protein-sorting events occur in 40–60-nm, often coated, membrane subdomains, which are below the detection level of the light microscope and are distinguishable in the EM only under the most favourable conditions. The TOKUYASU CRYOSECTIONING TECHNIQUE¹¹, which was introduced in 1973, has been improved and perfected so that it now generally surpasses other techniques with respect to membrane visibility and labelling sensitivity^{12–14}. FIGURE 2f and 2g show examples of a multivesicular endosome and small transport carriers, respectively, as they appear in ultrathin cryosections. The preferred tag for the visualization of antibodies by EM is colloidal gold (FIG. 2d–g), which can be

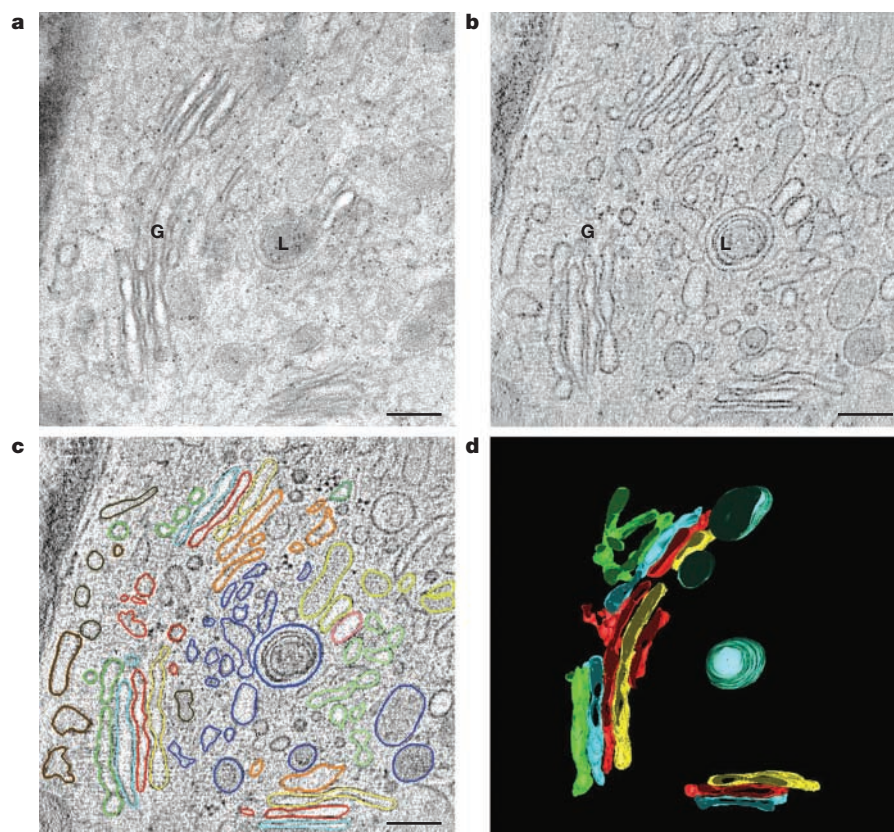


Figure 1 | The electron-tomography process on a 250-nm thick section of a high-pressure frozen, freeze-substituted and Epon-embedded dendritic cell. a | A digital image at 0° tilt, which was taken from a tilt series of 131 images that ranged from -65° to +65°. Because of the thickness of the section, the membranes near the Golgi complex (G) are poorly distinguished. The non-specific 10-nm colloidal gold labels (black dots) were deposited on the surface of the section to allow image alignment during the reconstruction process²⁷. L, lysosome. **b** | A 3.2-nm thin digital slice from the tomogram that was computed from the above-mentioned tilt series. The limited thickness of the digital slice allows the membrane morphology to be seen more clearly. **c** | Computer-assisted contour drawing on the digital slice of the tomogram that is shown in part **b**. The scale bars in **a**, **b** and **c** represent 200 nm. **d** | A contour model of some of the structures that are visible in the tomogram shown in part **b**. This figure was kindly provided by W. J. C. Geerts (Department of Molecular Cell Biology, Faculty of Biology, Utrecht, The Netherlands) and J. L. Murk (Department of Cell Biology, University Medical Center Utrecht, The Netherlands).

made in distinct sizes for the simultaneous localization of different molecules in a single experiment. The applications are numerous. For example, transport carriers can be defined by their cargo and at the same time by the presence of specific machinery proteins¹⁵. Counting the individual gold particles allows the relative intracellular distributions of a protein to be assessed and, under strictly defined conditions, even absolute protein concentrations can be determined¹⁶. A drawback is that the gold particles do not penetrate into cells or sections. ULTRASMALL GOLD, however, does penetrate into the sample, which increases the number of labelled antigens^{17,18}. The potential of ultrasmall gold to label antigens throughout the section makes it a promising candidate for the development of combined immuno-EM-ET approaches (see below).

New developments in immunoEM

Cryofixation and immunolabelling. An important development for future EM studies will be to combine cryofixation with immunoEM. The technical challenge here is that sections must be thawed before the immunolabelling procedure can be carried out. So, cryofixed cells must, at some stage, be chemically fixed as well. Several groups are developing approaches with this aim. The combination of high-pressure freezing and freeze substitution has been successfully applied, but generally gives suboptimal membrane visibility and labelling efficiency and is therefore limited in its use. One promising approach is to collect and thaw thin sections of frozen material in a mixture of membrane-stabilizing agents and fixatives. This 'section fixation' approach yields excellent images and allows

determination of the subcellular localization of secretory proteins and even cholesterol^{19,20}. Moreover, because section fixation occurs at low temperature and is rapid, it will probably solve at least some of the alleged penetration problems of chemical fixation. Alternatively, a combination of high-pressure freezing/freeze substitution and subsequent rehydration of the cells or tissue is presently under development. This 'rehydration' procedure will allow high-pressure-frozen material to be subjected to the Tokuyasu cryosectioning technique with a minimized chance of artefacts that are caused by chemical fixation, and therefore combines the strongest points of the two methods (J. W. Slot, unpublished observations).

Localizing lipids. Protein-trafficking events crucially depend on membranes, but remarkably little is known about the *in situ* intracellular distribution of lipids. A significant problem for lipid localization by EM is that routine fixation procedures immobilize lipids insufficiently and cause their extraction and redistribution. The best results so far were obtained with chemically fixed and freeze-substituted cells^{21,22}. More recently, with some simple modifications, the Tokuyasu approach was adapted to localize cholesterol¹⁹ and successfully provided the first EM double-labelling of two lipids — cholesterol and bis(monoacylglycerol)phosphate²⁰. An increasing number of lipid-binding toxins are becoming available for use in EM detection. Combined with the methodological innovations, this gives us hope that in the near future the *in situ* distribution of more types of lipids will be assessed at high resolution. Moreover, it opens up the possibility of studying protein sorting in a way that can be directly related to the lipid environment.

Adding new dimensions to EM

Electron tomography. The complexity of cellular architecture goes beyond what we can derive from two-dimensional (2D) images in the thin (~70 nm) sections that are normally viewed in the electron microscope. Our best 3D models of cell organelles are obtained by looking at their 2D appearance in a series of thin sections. This approach, however, has a resolution in the z-axis direction that is no better than the section thickness. The recently emerging technique of ET is not limited by the section thickness and can be used to generate 3D images of subcellular structures — called TOMOGRAMS — with an xyz resolution in the 2–10-nm range^{23–25}. The power of ET is that it produces 3D images of cellular structures in a section that cannot be deduced from conventional 2D EM projection images in which they appear overlapped. An

REVIEWS

example of the tomography procedure is illustrated in FIG. 1, which shows the conversion of an ET image (FIG. 1a) to a computer-based 3D

model (FIG. 1d). Movies that further illustrate this procedure are available online (see [Movie 1](#) and [Movie 2](#) online).

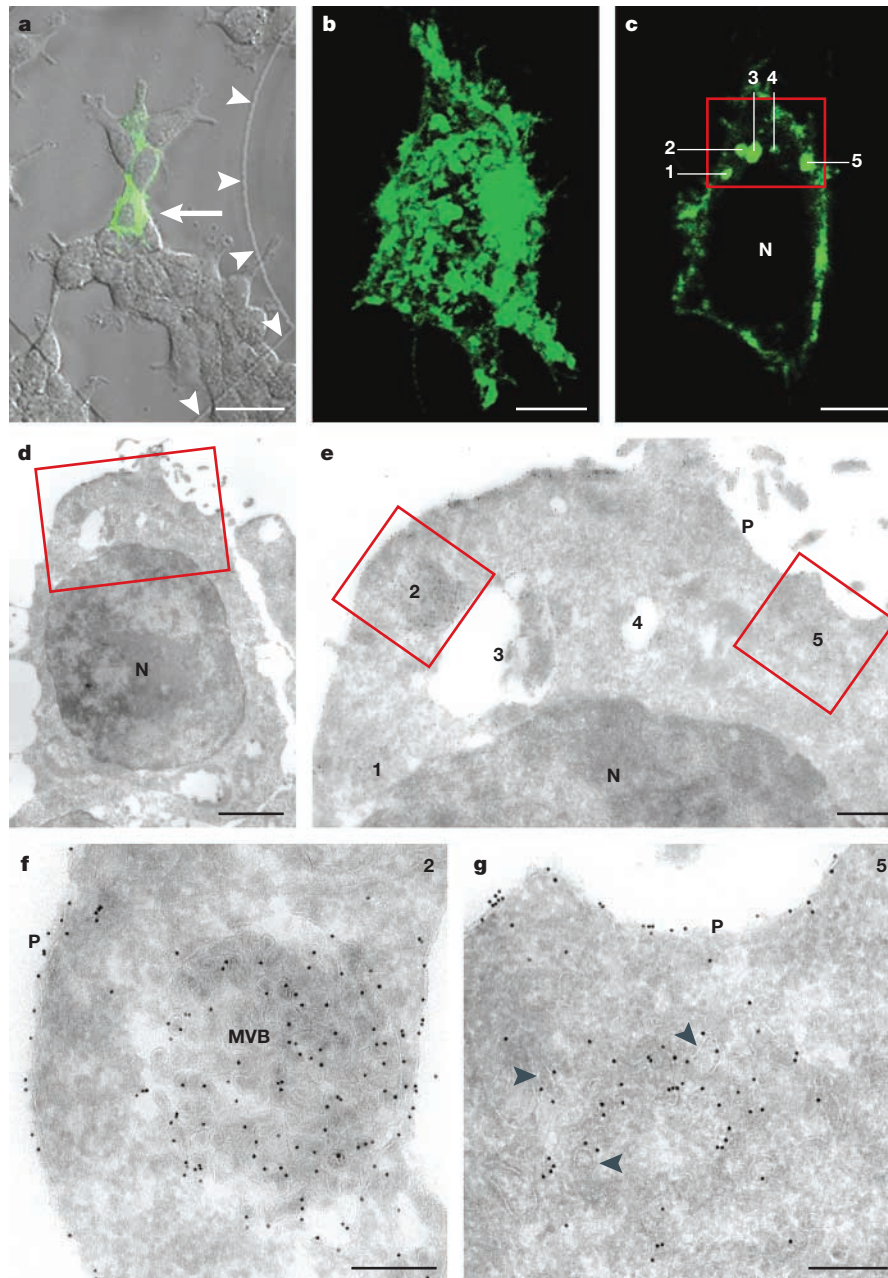


Figure 2 | An example of correlative live-cell-electron microscopy of a single cell. In this example, the fluorescent spots that are seen in live-cell imaging are exposed as being from distinct compartments using electron microscopy (EM). **a** | Confocal image of the selected cultured endothelial cell (arrow) that is expressing the endosomal-lysosomal protein CD63 conjugated to green fluorescent protein (GFP). Arrowheads point to marks on the coverslip (Eppendorf) that are used to relate back to these images from the cells under the electron microscope. **b** | A stacked fluorescence image of the selected cell. **c** | A confocal 370-nm slice of the selected cell that shows five distinct fluorescent spots. **d** | A correlative EM image of an ultrathin cryosection of the selected cell³⁹. The boxed area is the same as in part **c**. **e** | High magnification of the boxed area that is shown in **c** and **d**. The numbers refer to the spots that are shown in **c**, which are now highlighted by the presence of 10-nm gold particles (black dots) that were obtained by immunolabelling GFP. **f** | The original fluorescent spot 2 was found to correspond to a multivesicular body (MVB). **g** | The original fluorescent spot 5 was found to correspond to a cluster of vesicular profiles (arrowheads). N, nucleus; P, plasma membrane. The scale bars represent 25 μm (**a**), 5 μm (**b,c**), 2 μm (**d**), 500 nm (**e**) and 200 nm (**f,g**). This figure was kindly provided by V. Oorschot and R. Wubboldts (Department of Cell Biology, University Medical Center Utrecht, The Netherlands).

In the membrane-traffic field, ET is especially important for mapping compartmental boundaries in cells and to reveal the structure of complex membrane systems such as the endoplasmic reticulum (ER)–Golgi interface, the Golgi region and the endosomal vacuolar system. Tomograms of cellular organelles — such as mitochondria, the Golgi complex and peroxisomes — are being published at an increasing rate^{26–30} and have led to the reassessment of our understanding of some well-known cellular organelles. For example, ET has visualized a structural relationship between the ER and peroxisomes, which addressed a long-standing issue regarding peroxisome biogenesis³⁰. Moreover, ET has provided support for the *cis* to *trans* movement of entire Golgi cisternae²⁸, and has shown that post-Golgi transport carriers bud from multiple cisternae at the *trans* side of the Golgi complex^{26,27}.

With the development of high-quality charge-coupled device cameras and improved computer performance^{7,25}, automated ET has become a reasonably fast and well-controlled approach, as far as data collection in the instrument and image processing to produce a tomogram are concerned³¹. Translation of ET data into a biological model is still, however, less straightforward. For example, the computer-assisted drawing of membranes in a tomogram often involves the selection of structures and the subjective interpretation of the image. To assure the objectivity of a constructed model, it is therefore desirable that several independent researchers interpret the data that are embedded in the original tomogram.

ET and macromolecular imaging. Evidently, the power of ET would be increased tremendously if it were combined with immunoEM — for example, by using ultrasmall gold that penetrates and labels the full thickness of the sections. In several groups — including the combined efforts of our laboratories — immunolabelling protocols for thick sections are now being developed, but alternatives for immunolabelling are also emerging. Recently, the first ET results were obtained using direct cryoEM^{7,29,32}; this approach is referred to as cryoET. An important potential of cryoET is that, when the high-resolution structural template of a molecular structure is known (for example, from X-ray crystallography results) and the macromolecular structure is sufficiently large and distinct in shape, it can be identified unambiguously in the tomogram³³. Therefore, cryoET can be used to localize large supramolecular complexes in their cellular environment without the need for further steps, such as chemical fixation, staining,

labelling or genetic modification. For example, on the basis of its shape alone, the macromolecular structure of the 26S proteasome — the cytosolic multisubunit molecular machine that is responsible for intracellular protein degradation — could be identified in the tomogram that was constructed from a frozen *Dictyostelium discoideum* cell³². In the long term, using cryoET, we might be able to determine the cellular location of molecular complexes of only several hundred kDa on the basis of their shape, which opens up the exciting possibility of being able to photograph molecular machineries at work in cells.

Integrating live-cell imaging and immunoEM.

Imaging fluorescent proteins in living cells is a powerful technique that reveals the kinetics and direction of protein dynamics (see also the review on page S7 of this supplement). However, as with all fluorescent-microscopy methods, live-cell imaging lacks the fine-structure information against which the fluorescent signal can be interpreted. The resolution of light-microscopy-based methods is typically ~200–500 nm. Hence, a fluorescent spot can consist of many small vesicles or highlight only part of an organelle³⁴. By using immunoEM, proteins can be localized at the level of the smallest vesicle, however, the static EM images give no clue as to the directionality of the membranes observed. To fill the gap between live-cell imaging and EM, several groups are developing correlative-microscopy methods³⁵. In a first set of studies, green fluorescent protein (GFP) was genetically tagged to the G protein of the vesicular stomatitis virus (VSV-G)^{28,36}. The individual transport intermediates were monitored by video microscopy, after which the cell under study was fixed and processed for pre-embedding immunoEM using anti-VSV-G. This approach revealed an unexpected pleiomorphic complexity of the VSV-G transport carriers. A promising alternative method, which overcomes the drawbacks of the harsh pre-embedding immunoEM procedure, makes use of a new fluorescein derivative — ReAsH³⁷ (see also the review on page S1 of this supplement). With respect to what is important for correlative microscopy, when the fixed cells are intensely illuminated, ReAsH catalyses the photo-oxidation of diamino-benzidine, which results in electron-dense precipitates that can be visualized by EM³⁷.

Recently, a new preparation method has been introduced that allows Tokuyasu cryosections to be adapted for correlative microscopy³⁸. The power of this approach is shown in FIG. 2. Here, the intracellular trafficking of a GFP-conjugated construct of CD63 — a late endosomal–lysosomal

protein — was imaged by both confocal microscopy and immunoEM using anti-GFP–10-nm gold on thawed cryosections. FIGURE 2 highlights an important point: seemingly similar fluorescent spots (spots 2 and 5 in FIG. 2c) are localized by immunoEM to different organelles — that is, endosomes (FIG. 2f) and Golgi-associated structures (FIG. 2g), respectively. This example shows that fluorescent patterns must be interpreted with the utmost caution and that correlative high-resolution immunoEM can provide essential information on the structures that underlie these signals.

As well as establishing the kinetics and carriers of distinct intracellular transport steps, correlative live-cell–immunoEM will be particularly useful in studying those events that involve a sudden change in the cell. For example, the conversion of a multivesicular endosome into a tubular lysosome, which is a recently discovered aspect of antigen presentation by dendritic cells, has been described in live cells³⁹ and using immunoEM⁴⁰. Integrating this information would help to pinpoint the exact morphological changes and the molecular mechanisms that are involved in this intriguing process of membrane redistribution. For these correlative studies, it is important that the cells are fixed for EM at the exact moment at which the change takes place. High-pressure freezing is extremely rapid, but the time interval between cell selection under the light microscope and transfer to a high-pressure-freezing apparatus takes 15–20 seconds, which is too slow to fix rapid intracellular movements at the exact time of interest. Methods that solve this problem would provide another powerful tool for correlative live-cell–immunoEM/EM.

Finally, we would like to refer briefly to the potential of FLUORONANOGOLD complexes⁴¹. These probes can be used to label ultrathin cryosections and the fluorescent signal can be visualized using a sensitive light microscope. The same section can then be subjected to silver enhancement to amplify the signal for subsequent EM. At present, this technique has only been applied to fixed cells, but, when FluoroNanogold-labelled probes are designed that can be administered to living cells, it might potentially be extended to correlative live-cell–immunoEM imaging.

Final remarks

Some of the most profound insights in cell biology come from morphological analyses of subcellular structures. However, today, the appreciation of EM data is ambiguous. EM studies are often disdained as ‘descriptive’, as if

this were a weakness. The visualization of the interior of a cell in combination with molecular topography is a continuing inspiration for conceptual thinking and for understanding molecular function. Structure is unequivocally linked to function, and EM is one of the few techniques that can resolve the structure of subcellular compartments together with their molecular make-up. EM is no more descriptive than many of the genomics or proteomics approaches, it just operates at a higher level of biological organization.

The upcoming EM technologies will allow the localization of a progressively wider spectrum of molecules against the structural background of almost perfectly preserved cellular architecture. To develop a comprehensive view on healthy and diseased cells, future EM must stand side by side with complementary light-microscopy, genetic and molecular approaches. EM equipment has become increasingly user-friendly and, with the commercialization of many of the main reagents, EM applications have come into reach of every laboratory that is willing, and able, to invest resources. However, consultation with experienced researchers who are familiar with the morphological landscapes of the cell will be invaluable to ensure that data are interpreted accurately.

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REVIEW

MRI: volumetric imaging for vital imaging and atlas construction

Russell E. Jacobs, Cyrus Papan, Seth Ruffins, J. Michael Tyszka and Scott E. Fraser

Magnetic resonance imaging (MRI) is well known for its ability to capture non-invasively the three-dimensional structure of complex tissues such as the human brain. The physics underlying this technique means that it can be refined to collect high-resolution images in settings that would scatter the radiation used in direct-imaging techniques. This makes microscopic MRI a powerful tool to observe events and structures deep inside otherwise opaque soft tissues.

The success of reductionistic approaches in biomedical research has yielded an unprecedented knowledge of the components that are involved in biological processes, and researchers now face the challenge of integrating this knowledge into a more complete understanding of whole systems. For example,

the revolution in molecular biology has given us important new insights into the function of genes and gene products that might guide embryonic development. Many of the signalling pathways that are involved in the specification of cell types and the patterning of tissues are being defined, as are

the transcription factors that are involved in controlling cell-type-specific and region-specific gene expression. To answer the basic question of how an embryo develops, we must determine how these molecular processes are assembled into an organism. The classic publications in the field of experimental embryology^{1–3} illustrate the power of describing cell behaviours (in terms of cell lineages and movements) and then perturbing the development of an embryo to test hypotheses regarding the underlying mechanisms. Advanced imaging techniques offer an important stepping stone to integrate these disparate approaches⁴, and allow questions about cellular and molecular signalling events to be posed in the most relevant setting of the intact embryo.

Imaging thick specimens

The focus of intravital imaging is to follow cellular and subcellular events in the context of an entire organism. This presents a significant challenge to researchers, because all but the youngest embryos are significantly thicker than can be imaged easily with light. The image collected by a camera in a wide-field light microscope is typically blurred, because light from above and below the