Illumination of vertebrate development by fluorescence live imaging

Tadahiro Iimura D.D.S., Ph.D.1,2*， Mayu Sugiyama Ms.L.Sc.2,5,7， Yuji Makino M.D.2,6，
Ayako Nakane D.D.S.1,2,3， Takashi Watanabe D.D.S.1,2,4 and
Akira Yamaguchi D.D.S., Ph.D.1,2)

脊椎動物の発生におけるライブイメージング

飯村 忠浩1,2， 杉山 真由2,5,7， 牧野 祐司2,6， 中根 纜子1,2,3， 渡辺 高1,2,4， 山口 朗1,2

1) 東京医科歯科大学，グローバルCOE「歯と骨の分子疾患科学の国際教育研究拠点」
東京医科歯科大学大学院歯学総合研究科、2) 口腔病理学分野、3) 小児歯科学分野、4) 露顔面外科学分野
5) 東京薬科大学大学院生命科学研究科ゲノム情報学研究室、6) 順天堂大学整形外科学教室

Abstract
Development of organism is a dynamic but coordinated process that involves cell proliferation, migration and changes in cell function. Molecular biology and genome science promoted this realm of biomedical science by elucidating various common rules. Fluorescence live imaging has made it possible to quantitatively analyze multicellular process in 4 dimensions, thus providing coherent understandings of distinct levels of description form molecular levels to tissue, organ and organism. Application of this approach has given further insight and comprehension of dynamic process, not mere description of molecular hierarchy, into developmental biology and medicine. In this review paper, we, through introducing current topics in the body patterning, overview how the live imaging of fluorescent proteins has shed new lights on developmental biology.

Key words: morphogenesis, development, microscopy, fluorescence, imaging, fluorescent protein

*To whom correspondence should be addressed, iimura.gcoe@tmmd.ac.jp

1) Global Center of Excellence (GCOE) Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University
Sections of 2) Oral Pathology, 3) Developmental Oral Health Sciences and 4) Maxillofacial Surgery, Graduate School, Tokyo Medical and Dental University
5) Laboratory of Genome and Biosignal, Tokyo University of Pharmacy and Life Sciences,
6) Department of Orthopedic Surgery, Juntendo University School of Medicine
7) Laboratory for Cell Function and Dynamics, Brain Science Institute, RIKEN

抄録
個体発生は細胞の増殖や移動、機能分化が動的に協調して変遷していく過程である。分子生物学やゲノム科学の進展によって、脊椎動物の発生に関わる様々な共通ルールが明らかになってきた。蛍光ライブイメージングは、4次元での定量的な解析を可能にし、分子から細胞、組織、器官、個体までのより包括的な理解を助ける。これによって、発生・発達生物医学は、分子の階層的記述から、より動的に理解することが可能になってきた。本稿では、脊椎動物の発生・発達生物医学における最近のトピックをいくつか挙げ、その動向と今後の展望について議論したい。
Introduction

Embryonic development is a sequential and dynamic process in which cellular responses to patterning signals progressively restrict cell fate and localization into defined embryonic regions, which drives the growth and the shape changes of embryonic tissues. Distinct local signals induce cells to adopt specific behavior and functional potential that endow spatio-temporal organization of tissue development. Therefore, the morphological process in early embryonic development provides the fundamental framework of organogenesis.

Decades of study have established widely accepted conceptual frameworks for embryonic patterning and morphogenesis such as Spemann’s organizer, epithelial-mesenchymal interactions, and morphogen gradients. Advances in molecular biology and genetics have helped decipher the molecular cascades involved in these conceptual frameworks. However, providing new answers to old questions by modern technology is concomitant with raising new questions asked to be answered by more current methods. Mere uncovering of molecular cascades and genetic hierarchy appears to fall short of providing more satisfying understanding of the spatio-temporal dynamics of biological phenomena, including embryonic development. Recent advances in fluorescence microscopy have widened this view by providing coherent insights of different scales of descriptions from the molecular level to organogenesis and ontogeny.

The purpose of this review is to illustrate current views on how the bio-imaging of fluorescent proteins and probes has contributed to the field of developmental biology through introducing current topics in the body patterning. This technology has enabled the examination of molecular and cellular behavior to study their inherent dynamics in the three-dimensional environment of living multi-layered cell organism. The application of this technology has been extended to visualize the developmental processes of vertebrate embryo in four-dimensions.

Fluorescence protein imaging in the study of vertebrate development

The properties of a fluorescent molecule arise from a chemical moiety, the fluorophore, which absorbs light at a particular range of wavelength and subsequently emits light (fluorescence) at a specific range of longer wavelength. The major fluorophores commonly in use are small organic dyes, such as fluorescein, rhodamine and AlexaFlours, and inorganic nanocrystals, also known as quantum dots. They need to be conjugated to targeting molecules, such as antibodies, because of their limited cell permeability that restricts their applications especially in targeting intracellular molecules, even though they have advantages over fluorescent proteins such as the small size of organic dyes and the exceptional photosensitivity of quantum dots. In contrast to these fluorescent molecules, fluorescent proteins are genetically encoded and can be fused to any protein of interest. This makes their use protein-specific, minimally invasive and thus suitable for in vivo “live” studies.

Needless to say, a revolution in live fluorescent imaging has begun with the discovery of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Shimomura, et al., 1962), molecular cloning of GFP (Prasher, et al., 1992) and the expression of GFP in heterologous systems (Chalfie, et al., 1994) (Miyawaki, 2008).

GFP encodes a tripeptide within its own structure that is buried at the heart of a 2.4-nm beta-barrel and undergoes an autocatalytic reaction to form a functional fluorophore without any specific exogenous factors other than molecular oxygen. The discovery and development of fluorescent proteins have fostered various biomedical fields. Developmental biology is one of the fields that have benefited greatly from fluorescent protein technology.

From cellular dynamics to embryonic morphogenesis

Cell migration plays a major role during embryonic morphogenesis. Visualization of labeled cells using fluorescent proteins has enabled the real time analysis of cell migration in vivo.

Cell migration during vertebrate gastrulation

Gastrulation is a complex 3-dimensional process in which cells change position in the plane of the tissue, thereby producing the different germ layers and also providing the fundamental architecture of the embryonic body. Epiblast cells move, converge towards and ingress through a structure called the primitive streak in chick embryos, and ultimately migrate away from that streak.
The electroporation of GFP into epiblast cells and into a subset of primitive streak cells led to the characterization of the migration trajectories of the epiblast, with endodermal and mesodermal cells emerging from the primitive streak at different locations (Yang, et al., 2002). Live imaging showed that FGF4 and FGF8 behave like attractant and repellent growth factors, respectively. Combined imaging with fluorescently immuno labeled fibronectin: an extracellular matrix (ECM) and epiblast cells during primitive streak formation showed that, epiblast cells move little with respect to the ECM, which supports the notion that the majority of epiblast cell movement is associated with ECM migration (Zamir, et al., 2008).

Applying this chick live imaging with two color fluorescence cell labeling revealed a dual mode of mesoderm formation (Iimura and Pourquie, 2008, Iimura, et al., 2007). This observation indicated that the medial mesodermal structure is derived from a small portion of stem cells located in the epiblast adjacent to the Hensen’s node: the chick organizer; whereas the lateral mesodermal cells are derived from the continuous ingestion of epiblast cells, thus suggesting that the gastrulation mechanisms are better conserved between lower vertebrates and amniotes than previously thought.

Intrinsic molecular control on the timing of mesoderm formation by Hox genes

Collinear activation of Hox genes regulates the timing of mesoderm formation by controlling the flux of epiblast cells to the primitive streak during gastrulation (Iimura and Pourquie, 2006). Labeling groups of mesoderm precursor cells in the epiblast with different colored fluorescent proteins made it possible to monitor effects of different Hox genes expressed in these cells with unprecedented spatiotemporal resolution. This observation demonstrated unique roles of Hox genes in mesodermal cell distribution along the anterior-posterior embryonic body axis. This mechanism could thus provide a framework to understand the diversification of the axis formula among vertebrate species because the functional relevance of Hox genes on embryonic body plan and genomic organization has been demonstrated, especially in vertebrate evolution, (Iimura, et al., 2009, Iimura and Pourquie, 2007).

Real-time imaging of oscillatory signaling for embryonic body patterning

Body patterning of the developing vertebrate embryo is controlled by an ultradian oscillator, known as the segmentation clock. This molecular clock outputs periodic Notch, Fgf and Wnt signaling during embryonic mesoderm segmentation, known as somitogenesis, which endows the basic segmental patterning during organogenesis in the vertebrate body, including the vertebrae, and their associated muscle, tendon and nervous system. The analysis of transcriptional oscillations of the clock genes has been demonstrated by classical in situ hybridization methods on distinct stages of embryos because of the short oscillation periods, such as at, 30, 90 and 120 minutes for zebrafish, chick and mouse, respectively. It is, however, virtually impossible to analyze the phenotypic effect on this clock machinery, such as periodicity and amplitude. The recent development of fluorescent tools to study the dynamics of this oscillator represents a key advance in this field.

The promoter of the cyclic gene lunatic-fringe (Lfng) was fused to an destabilized version of the fluorescent protein Venus, and this reporter construct was used to generate transgenic reporter mice (Aulehla, et al., 2008). Despite the brevity of the oscillation period in comparison to the folding time and half-life of the Venus protein, this reporter successfully detects the oscillatory waves in cultured mouse embryos. The expression of the reporter reflects the periodic Notch response, since Lfng is a Notch target. This reporter mouse could be a powerful tool to test cross-talk among clock signals. Two-photon microscopy employing this strategy ruled out the possibility that constitutively active Wnt signaling altered the oscillation amplitude, but not the period, thus suggesting that periodic Wnt signaling controls Notch oscillations.

Probing cellular dynamics and embryonic development

Among the most important advances in laser microscopy is the capability for optical sectioning, which allows observation deeper inside tissues without interference from out-of-focus light and scatter, thus enabling unprecedented spatial and temporal resolution. This advance is achieved by the development of highly sensitive detectors and the engineering of fluorescent labeling.
The development of fluorescent cell cycle indicators has enabled biologists to visualize the cell-cycle behavior of individual cells within multicellular structures.

**Fuuci: fluorescent ubiquitination-based cell cycle indicator**

Sakaue-Sawano et al. recently developed Fuuci (fluorescent ubiquitination-based cell cycle indicator), a genetically encoded indicator for cell-cycle progression (Sakaue-Sawano, et al., 2008). This indicator system applies the regulation of cell-cycle-dependent ubiquitination of two cell cycle proteins, Cdt1 and geminin. Cdt1 and geminin have opposite effects on DNA replication during the S phase, and their protein levels oscillate accordingly throughout the cell cycle. Cdt1 protein levels are highest in the G1 phase just before DNA replication and decrease as cells transition into the S phase, whereas geminin protein levels rise during the S phase and fall during the G1 phase. Therefore, each of these two proteins appears once in distinct phase(s) of a single cell cycle in a reciprocal manner.

The original Fuuci probe was generated by fusing mKO2 (monomeric Kusabira Orange2) and mAG (monomeric Azami Green) to the ubiquitination domains of human Cdt1(hCdt1) and human geminin (hGem), respectively (Figure 1a). These two chimeric proteins accumulate in transfected mammalian cells during the cell cycle, reciprocally labeling nuclei of G1 phase cells in orange and those in S/G2/M phase in green. Therefore, these proteins function as effective G1 and S/G2/M markers. Furthermore, generation of transgenic mice that express Fuuci in every cell enabled the characterization of the cell-cycle behavior of embryonic neural progenitor cells and the cell-cycle properties of cancer cells both in vitro and vivo.

**Live imaging of cell cycle progression in a developing whole embryo**

Sugiyama et al. used the Fuuci system to successfully acquire a 4-dimensional view of cell cycle progression in developing zebrafish embryos (Figure 2 and 3) (Sugiyama, et al., 2009). The Zebrafish embryo is the most suitable target for live imaging using laser microscopy because of their external development at room temperature enables proper embryonic development under a microscope without any specific incubator, and its transparency allows feasible microscopic access to almost every stage of embryogenesis.

Transgenic zebrafish lines containing the ubiquitous hspa8 promoter were initially used to determine whether mammalian Fuuci constructs properly indicate cell cycle in living zebrafish. This assay indicated that Cdt1 is not...
and decrease as cells transition into the S phase, whereas during the G1 phase. Therefore, each of these two progeminin protein levels rise during the S phase and fall are highest in the G1 phase just before DNA replication proteins appears once in distinct phase(s) of a single cell cycle in a reciprocal manner. Cdt1 protein levels during the S phase, and their protein levels oscillate accordingly throughout the cell cycle. Cdt1 protein levels accumulate in transfected mammalian cells during the S phase, and geminin (zGem) were constructed, and then characterized using cultured fish cells, and constructed transgenic zebrafish lines. The fish-version of Fucci (named Cecyil: cell cycle illuminated) allowed observation of the dynamic patterns of cell-cycle progression in developing whole embryos. This observation illustrated intrigue correlations between cellular behavior and cell cycle progression, such as in the developing retina and notochord.
Local versus global cell behaviors

What is the behavior and contribution of individual cells within a cohort of migratory cells, and what is the morphogenetic driving force for directional growth of an embryo? The combination of fluorescence imaging and mathematical modeling provided unique answers to this question (Benazeraf et al., 2010). Combined imaging of fluorescently immuno-labeled fibronectin in posterior mesoderm cells during embryonic body elongation demonstrated that the gradient of random cell motility downstream of FGF signaling controls posteriorly directed tissue elongation in the amniote embryo. This work uncovered the unique biological phenomenon that tissue elongation is driven by the collective regulation of graded, random cell motion rather than by the regulation of directionality of individual cellular movements or migration.

Conclusions and perspectives

The application of molecular biology and genetics to embryology led to the discovery of conserved rules of construction in the animal body plan, such as molecular hierarchy, signaling and cross talk. It is thus considered to be important to recognize that current models of how cells respond to signaling pathways in vivo are almost completely devoid of any quantitative information. Little is known about the sensitivity, amplification, persistence, flow and fluctuation characteristics of signaling pathways and transcriptional responses. Detectable fluctuations and changes in oscillation at the molecular and cellular levels could be associated with reproducible cellular patterns for development and even with pathological states. Such variability or diversity has often been ignored to deduce simple rules, models and even understanding or consensus. The use of fluorescent proteins, for biological imaging promotes the quantitative analysis of developmental processes because their intensity and turnover can be measured. Adopting these quantitative studies in three- or four-dimension analyses is not merely an aesthetic improvement in imaging. This type of imaging has revealed the complexity and broadened our comprehensive understanding within a coherent framework of distinct levels of description. These techniques have benefited from the insights of mathematics, physics, engineering and computer science to build and test predictive models. The understanding will move forward through collaboration among these different fields of scientific research.

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