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Current Opinion in Cell Biology

Direct quantitative perturbations of physical parameters *in vivo* to elucidate vertebrate embryo morphogenesis

Soichiro Kato and Asako Shindo

Physical parameters such as tissue interplay forces, luminal pressure, fluid flow, temperature, and electric fields are crucial regulators of embryonic morphogenesis. While significant attention has been given to cellular and molecular responses to these physical parameters, their roles in morphogenesis are not yet fully elucidated. This is largely due to a shortage of methods for spatiotemporal modulation and direct quantitative perturbation of physical parameters in embryos. Recent advancements addressing these challenges include microscopes equipped with devices to apply and adjust forces, direct perturbation of luminal pressure, and the application of microforces to targeted cells and cilia *in vivo*. These methods are critical for unveiling morphogenesis mechanisms, highlighting the importance of integrating molecular and physical approaches for a comprehensive understanding of morphogenesis.

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Introduction

During development, dynamic cell movement is crucial in shaping the body, frequently using various physical parameters. The physical parameters in a tissue originate from cytoskeleton, cell adhesions, and extracellular matrix, as well as from non-cellular elements like substances filling a lumen. For instance, actomyosin can contract cell cortices, leading to changes in cell shape or movement [1,2], while aquaporin and Na^+/K^+ channels can alter the volume of internal fluid within a lumen, affecting the pressure therein [3]. Studies in mechanobiology have advanced our understanding by linking physics with developmental biology and show that physical parameters are involved in the process of morphogenesis.

Our understanding of the role of physical parameters in morphogenesis remains limited due to the lack of standard methodologies for their direct and quantitative perturbation *in vivo*. Currently, perturbations of physical parameters are primarily achieved through molecular manipulations, such as inhibiting actomyosin contractility or knocking down/out channels or transporters. However, researchers often tolerate the possibility that the manipulated molecules may be critical responders to the targeted physical parameter. Therefore, direct perturbation of physical parameters, which we define as an intervention using physical methods, is ideal for identifying their roles in morphogenesis. Moreover, perturbing physical parameters in specific spaces within embryos has been challenging, because the targets are either too small or too deeply located within embryos. Even when equipment successfully reaches the intended target, accurately assessing the extent of perturbation applied presents a new challenge. This necessitates the selection of appropriate tools and methods for the targets or the creation of custom equipment tailored to meet specific needs.

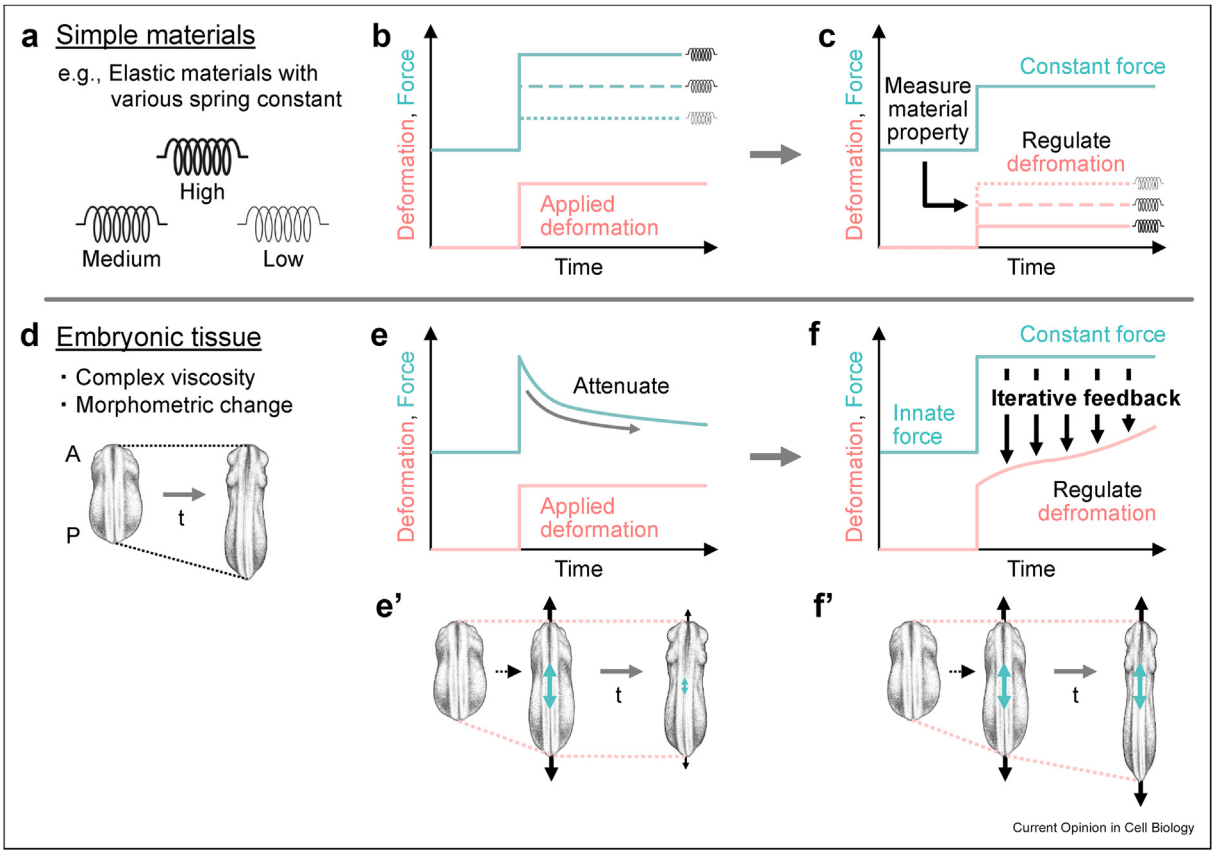
In this review, we summarize recent advancements in techniques and methodologies for the direct and quantitative perturbation of physical parameters within developing tissues of vertebrates *in vivo*. Specifically, we focus on the forces that have been successfully measured and perturbed in recent developmental biology studies: forces generated through tissue–tissue and cell–cell interactions, intraluminal pressure, and fluid flow. We also focus on other interesting physical parameters, temperature, and electric fields, as possible regulators of vertebrate morphogenesis. Lastly, we explore emerging technologies that hold promise for advancing the direct quantitative perturbation of each physical parameter *in vivo*, assessing how these technologies might address current challenges in developmental biology research.

Forces exerted by tissue or cell interplay

Direct quantitative perturbation is particularly crucial for elucidating the role of forces in embryonic development, given the viscoelastic or plastic properties of tissues and embryos [4–6]. The primary method for perturbing internal forces across multiple cells includes macro-scale stretching of tissues adhered to polydimethylsiloxane (PDMS, silicone polymer) substrates [7,8]. Also, micro-scale stretching of cell membrane can be achieved using optical tweezers affixed to cell membrane [9]. However, the forces within cells and tissues may differ depending on their viscoelastic properties and morphometric changes during development (Figure 1). The forces exerted on simple materials after deformation should be influenced by their material properties (e.g. elasticity) (Figure 1a, b), suggesting that the applied deformation must be adjusted to maintain constant forces among the various materials (Figure 1a, c). Embryonic tissues undergoing morphogenesis present additional complexity, owing to their viscoelastic properties and continuous morphometric changes (Figure 1d), as visualized by the Brillouin microscope [10]. In cases where their viscoelasticity follows the Maxwell model [4], the embryos and tissues are capable of attenuating forces through their deformation (Figure 1e, e') [11]. Consequently, to achieve constant forces in tissues, the deformation must be applied repeatedly (Figure 1f, f'). Therefore, in the force perturbing experiments, it is preferable to continuously monitor and adjust the applied deformation by providing iterative feedback [6,12].

Such a feedback approach is ideal for investigating the role of intercellular and intertissue forces during morphogenesis, especially for long-term force

Figure 1



Concept of direct quantitative perturbation of forces within simple materials/embryonic tissue **(a)** Schematic of an elastic spring representing simple materials. Thicker lines indicate less deformable (higher spring constant). **(b, c)** Time series schematic diagrams showing the applied deformation (pink line) and the internal forces of the material (blue line). Solid, dashed, and dotted lines represent the forces generated after applying the same deformation in materials with high, medium, and low spring constants, respectively **(b)**. The deformations required to generate a constant force are represented by solid, dashed, and doted lines in each case **(c)**. **(d)** Schematic of *Xenopus* embryos as examples of embryonic tissue, depicting complex viscous properties and morphometric changes during development, characterized by elongation along anterior–posterior axis. A: anterior, P: posterior. **(e, f)** Time series schematic diagram of applied deformation (pink line) and internal forces in the embryo (blue line) **(e, f)**, accompanied by representative schematic showing the deformation and forces in the embryo **(e′, f′)**. The applied force by deformation attenuates due to viscosity and morphometric changes **(e, e′)**, necessitating iterative feedback between force and deformation through monitoring both values for long-term application of constant force in embryonic tissue **(f, f′)**. Xenopus illustrations ©Natalya Zahn (2022), Xenbase (www.xenbase.org RRID:SCR_003280), [81].

applications that can last from minutes to hours. Some molecular-based sensors, including FRET-based force sensors, are valuable tools for monitoring applied forces [13]. If force application is coupled with the methods of visualizing forces, the feedback approach becomes feasible, although it is still challenging to combine independent methods for measurement and perturbation. Recently, a feedback-based apparatus has been developed to apply forces to embryos over periods ranging from minutes to hours. The Tissue Force Microscopy (TiFM) system, equipped with closed-loop feedback control for applying forces in chick embryos, has been introduced [14]. This system is designed for small tissues ranging in size from 100 μm to 1 mm and successfully measured axial elongation force within a range of 40–100 Pa. Applying forces within the "in vivo" range facilitates body elongation by causing specific cell movement, demonstrating that the magnitude of endogenous forces is critical for the elongation speed. *In situ* monitoring of force application with this system will enhance our understanding of key mechanisms, such as the role of cell density in the rate of morphogenesis [15].

Recent technological advancements have enabled the development of customized apparatuses designed to apply forces on viscoelastic tissues and embryos. Previously, complex analog circuits were necessary to control actuators for feedback and forces adjustment. Now, microcontrollers and single-board computers, such as Arduino and Raspberry Pi, can replace that expertise and complex systems [16–18]. These microcontrollers can be integrated with robot-assisted tissue micromanipulation for procedures like microsurgery of embryos [19], as well as with force sensors and microindenters [20]. Commercially available 3D printers have become a powerful tool for producing custom-designed equipment tailored to specific experimental setups [21,22]. Recent high-end models can print objects with microscale precision, enabling the creation of intricate components that can be seamlessly integrated with glass needles, tungsten probes, and optical fibers, essential for the precise measurement and perturbation of tissue stress. More recently, objects can be printed even inside embryos, allowing for more effective measurement of tissue force [23]. Adopting such new techniques to perturb forces, alongside routine functional analyses of molecules, will enhance the discovery of mechanisms underlying morphogenesis.

Force exerted by intraluminal pressure

Investigation into the role of intraluminal pressure in morphogenesis has significantly progressed through strategies that incorporate both indirect and direct measurements and perturbation of pressure [24,25]. Despite the potential influence of the material properties of surrounding cells on these measurements, intraluminal pressure has been estimated by observing deformation of hydrogel encapsulating an embryo [26] or displacement of microbeads attached to luminal tissues [27]. Inserting a glass needle connected to microelectrodes or electric sensors into the lumen is the most reliable method for accurately measuring minute pressures in embryos. Using these tools, the influx of luminal fluid into the needle can be converted to electrical resistance [25,28], sensor strain [24], or deformation of water–oil interface inside the glass needle [29]. However, such direct measurements of the pressure are only feasible when the target cavity is sufficiently large to accommodate the insertion of glass capillaries. Currently, the internal pressure of small cavities, such as intracellular gaps within tissues, is estimated using Laplace's equation based on the shapes of cell outlines [30,31].

Recent advances in the direct and quantitative perturbation of luminal pressure are opening a new area of research in morphogenesis, following previous studies that have largely relied on indirect strategies targeting molecules. Ouabain is commonly used to decrease intraluminal pressure by inhibiting Na^+/K^+ ATPase [3,32]. Another method involves *Clostridium perfringens* enterotoxin (C-CPE), which reduces fluid accumulation within the lumen by attenuating tight junction sealing [33]. Traditionally, luminal pressure has been directly perturbed by connecting internal spaces to the external via capillaries [34,35]. These approaches have contributed to identifying the role of pressure in normal morphogenesis, despite the lack of information on the pressure amount they disturbed. More recently, it has become possible to assess the pressure resistance of luminal tissues by injecting a defined volume of fluid into the lumen and continuously monitoring the pressure. One significant finding from this method is the identification of gating mechanisms of the blastopore of *Xenopus* embryos, which, although not a focus of previous studies, are crucial for the fluid movement essential for normal morphogenesis [28]. In the future, the development of a new apparatus for quantitatively manipulating luminal pressure, which incorporate technologies like single-board computers, may potentially catalyze a paradigm shift in the focus of morphogenesis research [36].

Given that luminal pressure is determined by the balance between osmotic pressure and the tension of the luminal wall, osmotic pressure within the cavity and tissues represents another variable that requires measurement and perturbation. Previously, osmolarity was assessed invasively, necessitating the collection of fluid from embryos [37]. Recent innovations may enable the direct perturbation of osmotic pressure *in vivo*. By employing a double emulsion droplet with a size of approximately 30 μm, osmotic pressure in early zebrafish embryos was successfully measured [38]. In the future, this technique could be used for monitoring osmotic pressure by combining it with the injection of a specific buffer, thereby enabling quantitative perturbation.

Force exerted by fluid flow

Fluid flow has been recognized as a regulator of morphogenesis, as evidenced by discoveries of left-right axis determination through cilia-dependent nodal flow [39]. Blood vessel formation is another example controlled by mechanical stress from blood flow [40]. The dynamics of flow have been analyzed by visualizing the movements of fluorescent beads and examining the morphology of the luminal interface [41,42]. Such a measurement method has revealed the flow velocity within tissues. For example, mouse node cilia generate flow ranging from 1 to 2 μm/s [43], whereas the cilia in zebrafish Kupffer's vesicles (KV) generate flow between 5 and 10 μm/s [44]. Blood flow in zebrafish embryos is significantly faster, ranging from 100 to 1500 μm/s [45,46]. Perturbations of these fluid flows are effectively induced through various methods, including the mutation of cilia genes to inhibit their motion [47], the addition of methylcellulose to increase fluid viscosity and slow down the flow [41,43], and the manipulation of flow using both chemical and mechanical approaches [48,49]. These perturbations demonstrated that fluid flow itself controls morphogenesis.

Recent advancements in optogenetic, magnetic, and acoustic technologies have enabled targeted perturbation of cells and cilia responding to fluid flow. Disturbing the flow still leaves unsolved the question of whether chemical transport or physical force transmission is the primary contributor. To address this uncertainty, direct application of force to the target, including bending individual cilia using optical tweezers, has been conducted [50,51]. These studies demonstrate that the physical force to bend cilia is a key in determining the left-right axis. Interestingly, the force required for bending cilia appears to be specific to species or tissues. For instance, recent papers reported that mouse node cilia require 12 pN [50], while zebrafish KV cilia require 0.6 pN [51] to bend. It is also noteworthy that these magnitudes of force do not correlate with the flow velocity described above. In studies of vasculogenesis, pressing magnetic beads or microbubbles onto endothelial cells with micrometer precision, controlled by external magnets and acoustics, contributes to understanding the role of physical parameters [52,53]. A significant advantage of these remote manipulations is their ability to target tissues located in deeper layers of embryos. This remote control of micro-perturbation distinguishes between the roles of molecules and physical forces, shedding light on longstanding questions about the mechanisms of left-right symmetry breaking and the formation of tubules and their appendages, such as cardiac valves.

Methodologies for micro-perturbing fluid flow at specific locations will enhance our understanding of the relationships between flow and forces. Soft robotic microsystems, controlled by acoustics, may emerge as new tools for directly perturbing local fluid flow [54,55]. Such microscale devices, fabricated from polymers, may be inserted into the lumina of embryos to perturb the internal flow acoustically. Although challenges related to their size (150–300 μm) and potential toxicity remain [56,57], integrating non-biological material and tools could pave the way for innovative methods to directly and quantitatively perturb fluid flow.

Temperature and electric fields

Temperature and electric fields are distinct physical parameters that possibly regulate morphogenesis independently from the physical forces we described above. It is well-established that temperature influences the rate of development, exemplified by its application in studies on the resynchronization of cell cycle in *Xenopus* embryos [58]. Beyond the effects on developmental rate, increasing environmental temperature from 14.5 °C to 26.5 °C was shown to alter gene expression that regulates the number of motor neurons in *Xenopus* embryos [59]. More recently, single-cell RNA-seq analysis of zebrafish embryos has revealed heterogeneous temperature sensitivity across tissues, with the notochord being particularly susceptible to higher environmental temperature up to 34 °C [60]. Notably, similar findings in *Ciona* identified the notochord as the tissue most distinctly disrupted by high-temperature stress around 25 °C [61], suggesting a broader role for temperature in regulating specific tissue morphogenesis.

Electric fields are another significant physical parameter capable of regulating morphogenesis, characterized by their ease of perturbations from the environment [62,63]. It has long been established that neural crest cells cultured from amphibian and avian embryos exhibit electrotaxis, the directed movement of cells in response to an electric field [64,65]. More recently, electrotaxis has also been observed in mouse neural crest cells, although their directionality is opposite to that seen in amphibians and avians [66]. Efforts to directly measure endogenous electric fields on the surface of amphibian embryos using vibrating microelectrode have provided support for the role of electrotaxis in guiding cell migration during development [67]. Importantly, adult epidermal cells in fish similarly respond to electric fields that are generated by wounds [68]. Not only directional cell migration, but electric fields also regulate the three-dimensional structure of cultured cysts, altering its size and shape [69]. These findings were achieved by simply applying an electric current beneath the cells and tissues.

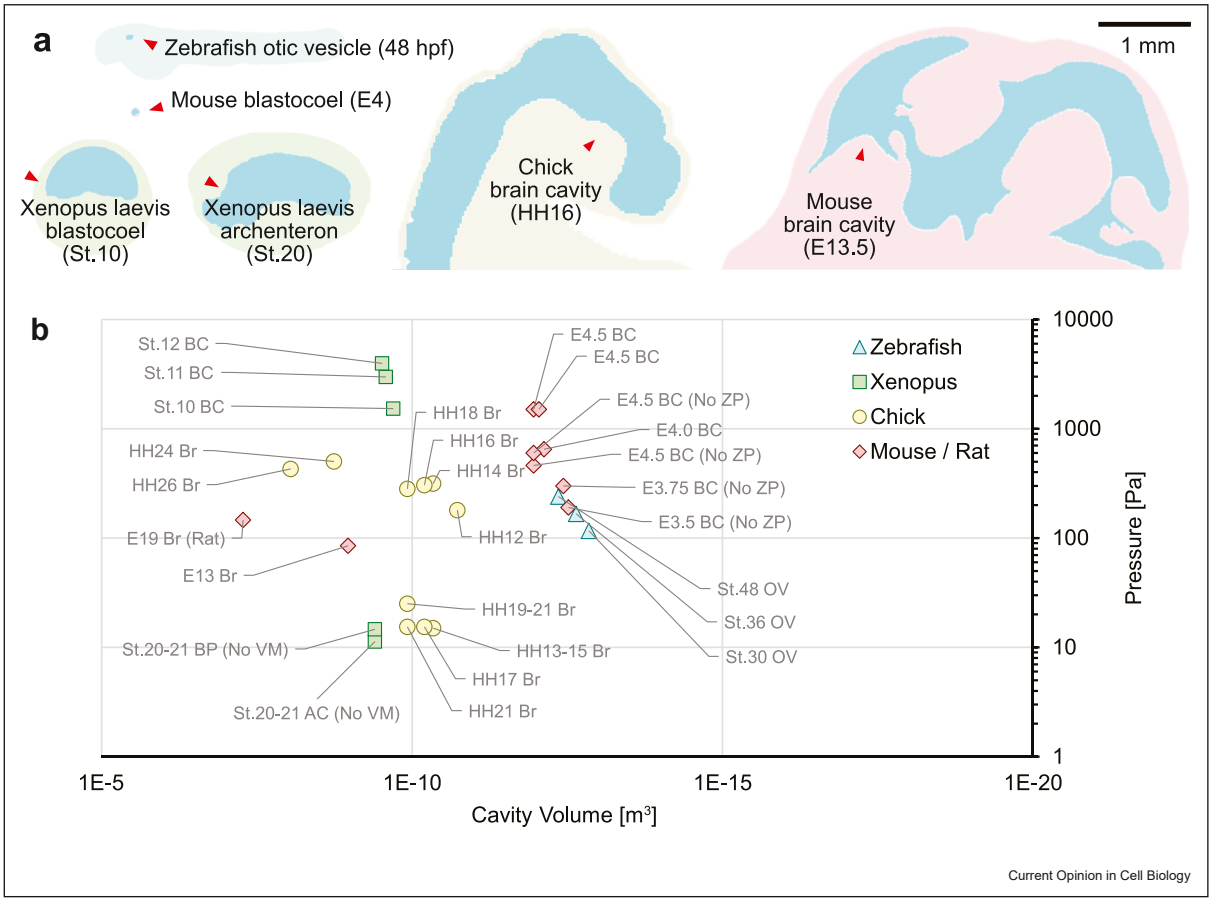
While external perturbations of temperature and electric fields are feasible, the methodologies for accurately quantifying their effects within tissues still require further refinement. Notably, calorimeter-measured heat flow during the cleavage stage of zebrafish development

demonstrates oscillation that correlate with cell division [70]. This finding prompts further investigation into whether temperature influences morphogenesis by quantified perturbations of internal temperature. Although current technical limitations in measuring internal temperatures in embryos hinder effective quantitative perturbation, advancements in techniques such as optical heaters and thermometers are expected to address these challenges [71,72]. Considering the sensitivity of specific tissues to temperature stress [60,61], precise spatiotemporal control of measurement and perturbation could facilitate the identification of temperature-responsive targets. Such micro-quantitative perturbations are also essential for studies exploring the role of electric fields on morphogenesis.

Discussion

Direct and quantitative perturbation of physical parameters *in vivo* is essential for understanding their roles in morphogenesis; however, unlike well-established methods such as knockdown and knockout techniques used to disrupt gene expression, strategies and frameworks for such perturbations of physical parameters are not yet well defined. This lack of standardization can result in variability of results depending on the operator

Figure 2



Relationship between volume and fluid pressure in embryonic cavities **(a)** Scale-aligned schematic of various embryonic cavities with pressures measured in previous studies [25,28,32,36,82–84]. Red arrowhead: fluid-filled cavity, scale bar = 1 mm. Abbreviations: hpf, hour post fertilization (zebrafish); St, Nieuwkoop-Faber stage (*Xenopus*); HH, Hamburger Hamilton Stage (Chick); E, Embryonic day (days post conception, dpc, Mouse). **(b)** Diagram showing the intraluminal pressure and volume of the cavity approximated as a sphere, based on values referenced from previous studies [24–28,32,34,36,85–90]. Horizontal and vertical axes of the double logarithmic chart represent measured or estimated cavity volume [m^3] and measured pressure [Pa], respectively. Note that there are no data for cavities smaller than 1E-15 m^3, implying another challenge in developing technologies capable of measuring and perturbing such small cavities. Blue triangles, green squares, yellow circles, red diamonds indicate the measured cavity pressures in zebrafish, *Xenopus*, chick, and mouse/rat embryos, respectively. Each point is labeled with the developmental stage and type of cavity using the following abbreviations: BC, Blastocoel; OV, Otic vesicle; AC, Archenteron; BP, Blastopore pressure resistance; Br, Brain cavity; VM, vitelline membrane (membrane surrounding *Xenopus* embryos); ZP, zona pellucida (membrane surrounding mouse embryos). For cavities where the volume is not precisely measured, volumes at similar stages or estimated from diameter or cross-sectional area, approximated as a sphere (*Xenopus* St.20-21 AC, BP (No VM)) or as a column (Mouse E.13 Br), are presented.

and the measuring instruments. It is clear that developing and adopting multiple methods for measuring physical parameters is necessary to obtain accurate values, which currently show variability (Figure 2). Additionally, the challenge of measuring over a certain duration along with targeting specific sizes frequently troubles researchers, necessitating the development of specialized equipment. In this review, we highlight recent advancements in techniques that enable the direct and quantitative perturbation of physical parameters *in vivo*.

One of the important questions for future research in this field is elucidating the relationship between force magnitude and both tissue size and morphology. Research has demonstrated that intraluminal pressure determines the size of the zebrafish otic vesicle and mouse blastocyst [24,25]. Our comparison of aggregated data from previous studies shows that, when approximated to a sphere for volume calculations, mouse blastocysts exhibit sizes and intraluminal pressures similar to those of zebrafish otic vesicles (Figure 2a, b: BC at E.3.5–3.75 and OV at St. 36–48). Additionally, developing chick brain and *Xenopus* archenteron without vitelline membrane exhibit similar sizes and pressures (Figure 2b: HH21 Br and St. 20–21 AC and BP). These may stem from a fundamental role of intraluminal pressure in orchestrating morphogenesis at distinct tissue scales. Exploring the direct impact of pressure by quantitative perturbation on specific luminal morphogenetic events, such as the twisting and branching of tubules, could lead to previously unrecognized mechanisms of morphogenesis.

Actual quantities of measuring and perturbing forces raise other important questions in this field. For example, with regard to the forces generated by tissue–tissue or cell–cell interplays, a significant challenge remains in linking well-characterized molecular-level forces, such as those exerted by myosin, to the deformation forces observed at the tissue level. It is unclear how the forces produced by individual myosin molecules, which generate only a few piconewtons, scale up to elongate tissues with forces reaching several micronewtons [5]. Due to this knowledge gap, it remains uncertain whether the accumulation and quantity of myosin at cell–cell junctions alone can generate the necessary forces for tissue elongation [73]. To investigate the potential mechanisms of force amplification from the molecular to the tissue level, it is crucial to conduct direct quantitative perturbations of physical parameters at each scale.

Lastly, a major challenge is to elucidate how the magnitudes of physical parameters relate to the molecular and physical properties of cells or organelles that enable them to sense these parameters. This is typically observed in the sensing of fluid flow by cilia, as we mentioned that flow velocity does not correlate with the forces required for bending cilia. This fact raises the possibility that the stiffness, length, or molecules on the cell membrane of the cilia might modify the mechanosensitivity [74]. Direct quantitative perturbation will help identify what is required for sensing physical parameters and also clarify the significance of our body design in generating the physical parameters.

Conclusion and future perspectives

Interest in the role of physical parameters in developmental biology began alongside attempts to measure and perturb them within embryos, coinciding with the rise of experimental developmental biology.

Since the late 19th century, disturbing gravity in embryos has been a classical method for demonstrating that physical parameters are involved in morphogenesis [75–77]. These studies used techniques such as placing the embryos upside down, rotating them with a clinostat, or sending embryos to space to perturb gravity at the whole embryo level. The equipment introduced in this review builds on these strategies, allowing for finer measurement and perturbation of forces at the tissue and cell level. Such advancements have significantly enhanced our understanding of how physical parameters contribute to morphogenesis. In the future, devising and crafting innovative measurement and perturbation devices will enable the identification of unexplored physical parameters regulating development. For instance, friction force at the tissue–tissue interface is one emerging physical parameter to be investigated [78–80], although its magnitude is unknown, and methods of perturbation have yet to be established. Ongoing exploration of such unexplored physical parameters, including temperature and electric fields, from a technical perspective, will deepen our understanding of the principles of morphogenesis.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used [ChatGPT/OpenAI] in order to improve language and readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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