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REVIEW

Microsystems for controlled genetic perturbation of live *Drosophila* embryos: RNA interference, development robustness and drug screening

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Abstract A combination of genetic regulation and microenvironmental perturbation might prove especially useful for identifying the fundamental mechanisms responsible for development biology and critical biochemical networks. This could transform our understanding of genomics, proteomics, and drug discovery and lead to personalized drugs and molecular diagnostics for improved healthcare. With the complete sequencing of the *Drosophila melanogaster*, there has been a growing interest in correlating its genetics to human disease. The *Drosophila* embryo is an excellent whole-animal model, and is ideal for high throughput analysis. This review highlights research on the recent development of miniature tools to study critical cellular processes in *Drosophila* embryogenesis. First, we discuss the use of micro-injectors to conduct chip-level RNA interference experiments on self-assembled *Drosophila* embryos in order to determine how genes contribute to the growth and function of a developing organism. Second, we review recent progress on perturbing the microenvironment as a complementary approach to perturbing the genetic components of the development network. We designed a bio-compatible microfluidic device that automatically positions live embryos within a fluid-filled imaging channel and exposes embryos to constant or biphasic temperature or different drug concentrations. We have demonstrated that a thermal gradient applied across live *Drosophila* embryos results in asynchronous cellularization. Additionally, preliminary drug screening experiments were carried out on live *Drosophila* embryos in both 96-well plates and microfluidic channels. Time-lapse differential interference contrast microscopic images were taken to show the effects of colchicine (399.40 Da) and cytochalasin D (507.62 Da) on cellularization.

Keywords *Drosophila* embryo · RNA interference · Microinjection · Drug screening · Microfluidic self assembly · Perturbation

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1 Introduction

In 1995, Edward B. Lewis, Christiane Nüsslein-Volhard and Eric F. Wieschaus won the Nobel Prize in medicine for their pioneering work in identifying genes that played a key role in the body plan, body segment formation and specialization in *Drosophila melanogaster* embryos (Lewis 1978; Nusslein-Volhard and Wieschaus 1980). This was the first successful demonstration of mapping genes regulating a cellular function in a complex organism. This discovery has since led to the mapping of many other regulatory responses in *Drosophila* embryos (Niemuth and Wolf 1995; Eldar et al. 2004; Ochoa-Espinosa et al. 2005). Originally, the *Drosophila* was used to study genetic inheritance (Rubin and Lewis 2000), but the complete sequencing of the *D. melanogaster* genome has fashioned

the basis for advanced functional studies in several fields, such as developmental biology (VijayRaghavan 2002; Anderson and Ingham 2003), cellular and molecular biology (Roll-Hansen 1978; Baker 1989), behavioral studies (Friedrich 2000; Perazzona et al. 2004), and human disease (Chien et al. 2002; Stelling et al. 2002; Buckingham 2004; Monzo et al. 2006; Bolduc et al. 2008; Tessier and Broadie 2008). The striking similarity of *Drosophila* defense response to disease and human immune response has become increasingly evident since fruit flies are 44% genetically similar to humans (Adams 2000; Bier 2005) and 62% of all human disease genes are conserved in the *Drosophila* genome (Fortini et al. 2000; Anderson and Ingham 2003). Sophisticated technologies have been deployed to augment further discoveries to precisely correlate *Drosophila* genetics and human disease (Chen et al. 2004; Zappe et al. 2006; Zhang et al. 2006; Dagani et al. 2007).

The technology for perturbing and observing cellular function is very important for gathering the data needed to understand the biomechanics, biochemical processes and their interactions in a developing *Drosophila* (Fig. 1). The most widely used method for exploring regulatory networks is injecting oligonucleotides (Carthew 2001; Agrawal et al. 2003; Buckingham 2004; Drysdale 2008) or other small molecules into a cell to inhibit important processes and induce cellular response. High throughput assays that are required for rapid identification of novel genes, and the instrumentation incorporated to study them, make the use of existing vertebrate animal models unsuitable. The model organism *Drosophila* allows in vivo genome-wide assays and high-throughput screens due to the availability of unmatched genetic techniques, the organism's transparency, and the ability to grow it in minute volumes (Rubin 1988; Rubin and Lewis 2000; Buckingham 2004, 2005; Manning 2006). Yet, since the first publications in early 1900s by Morgan (1929), few changes have been made in the way scientists manipulate this tiny organism, as a result of which even simple large-scale assays still take months to years to complete. Importantly, due to the lack of key technologies, several assays either cannot be performed at all or have to be dramatically simplified for high-throughput screens.

Micro-scale platforms (Microsystems, micro-nano electromechanical systems, or MEMS/NEMS, and micro-robotics) have emerged as practical tools for high throughput manipulation and perturbation of living *Drosophila* embryos (Yu and Nelson 2001; Bernstein et al. 2004b; Chen et al. 2004; Lucchetta et al. 2005, 2006; Dagani et al. 2007; Yu 2009). Such platforms have great potential in revolutionizing conventional embryo culture techniques through specifically engineered surfaces, structures and accurate spatiotemporal probing. Microfluidic technology, fabricated by soft lithography, has proven useful in providing methods to create and examine varied chemical combinations and their effects on cell viability and growth (Folch et al. 2000; Beebe et al. 2002; Voldman 2003; Hung et al. 2005; Khademhosseini

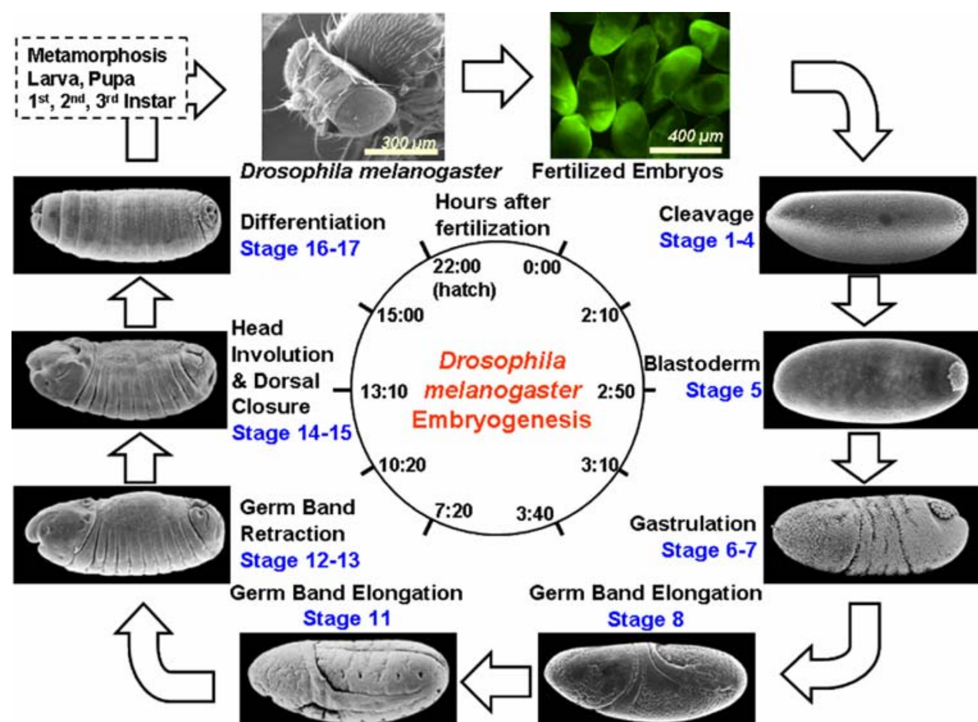


Fig. 1 *Drosophila* development and embryo morphogenesis. The process goes through many complex cell shape changes and movements. Initially, the fertilized egg divides many times and becomes a hollow ball of cells (cellular blastoderm). The posterior end of the embryo migrates up and over the top (dorsal surface) of the embryo, a process called germband extension, which is responsible for delivery of specific cell types to the interior of the embryo. The reverse process (germband retraction) then follows, leaving a large open hole on the dorsal surface of the embryo. Dorsal closure then takes place to remove the hole by zipping up the surrounding epidermis. At this point, the embryo has completed embryogenesis and is ready to proceed with hatching (Wolpert et al. 2002)

et al. 2006; Wang et al. 2007b; Ye et al. 2007; Kang 2008). However, it is increasingly apparent that understanding the development of living organisms will involve research not just at the molecular or cellular level, but also at a broader or whole system level (Love et al. 2004; Breslauer et al. 2006; Rohde et al. 2007; Zhang 2007). *Drosophila* embryos are ideal whole system models for developmental experiments. Recently, microfluidic devices have become more prevalent in *Drosophila* embryo analysis. For example, microfluidic devices were used to sort *Drosophila* embryos (Chen et al. 2004) based on certain traits, such as a specific genotype or mutation, at a particular stage in embryogenesis. Integrating efficient microfluidic sorting techniques with powerful detection technologies could give rise to faster and more precise embryo sorting systems (Furlong et al. 2001). Such high throughput sorting techniques, evaluated by time of analysis, accuracy and optimal yield and purity combinations, opens the door for large-scale preparations of embryos that are required for the study of gene expression during embryo development.

High throughput molecular screening (HTS) is the automated, simultaneous testing of thousands of distinct chemical compounds in models of biological mechanisms (Hertzberg and Pope 2000). Active compounds identified through HTS can provide the starting point for the design of powerful research tools that allow pharmacological probing of basic biological mechanisms in *Drosophila* embryos, and which can be used to establish the role of a molecular target in a disease process, as well as its ability to alter the metabolism or toxicity of a therapeutic agent. Microdevices have paved the way for innovative HTS methods to perturb live *Drosophila* embryos in large quantities. In general, high throughput perturbations performed on live embryos can be classified into two types: internal and external perturbation as shown in Fig. 2. *Internal perturbation* involves the use of genetic or pharmaceutical stimuli within the biological model system to affect the development. For example, microinjection has been used for the direct delivery of active elements into a developing embryo. *External perturbation* is the direct disturbance of the external environment surrounding a developing embryo. This is done using various stimuli, such as mechanical, thermal, fluidic and chemical cues, for the external probing of embryo viability and development.

In this paper, we review the recent progress in combining immobilization, perturbation and visualization within microsystems (including MEMS, and/or microfluidics) towards developing a high throughput screening method on *Drosophila* embryos. The technologies are interconnected towards achieving specific biological tasks such as RNA interference (RNAi) or studying development robustness, and drug screening. Both internal and external perturbation methods in the microscale will be discussed with respect to the three key engineering steps: immobilization, perturbation, and visualization. (1) Immobilization: A major bottleneck when performing HTS is the accurate placement of embryos on a functional platform. The manual placement of embryos is time consuming and could jeopardize time critical experiments that are common in *Drosophila* developmental biology. It is therefore advantageous to develop methods to automatically assemble embryos with deliberate positioning and orientation. (2) Controlled Perturbation: Following successful immobilization, embryos can then be precisely exposed to well-controlled physical stimulation or extreme micro-environmental conditions to produce alternations of biochemical functions. For example, laminar flows in microfluidic channels can generate precise gradients of drug solution, temperature, and shear force around an immobilized embryo (Lucchetta et al. 2005, 2006; Walker et al. 2004). In addition, coupled mechanical and optical probing on the micro-scale may be

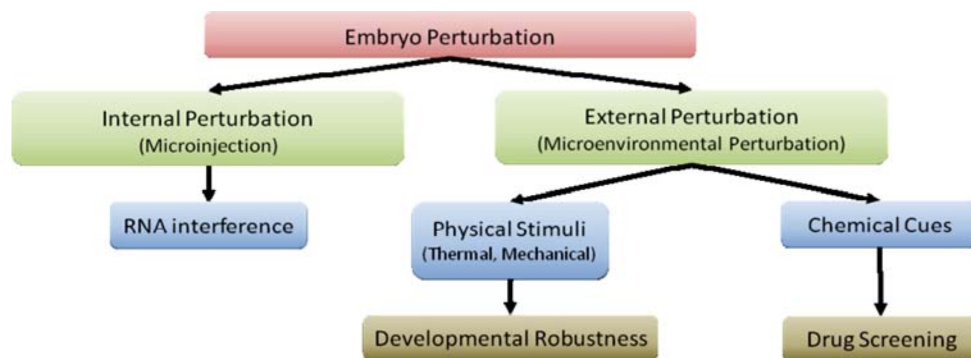


Fig. 2 Microtechnologies for high throughput analysis of live *Drosophila* embryos are interrelated. Key steps of micro-scale technologies that offer precise embryo analysis can be combined as shown to perform high throughput screens. Such screens will create new possibilities to investigate the impact of genetic variation and microenvironmental perturbation on intra-cellular biochemical signaling pathways, cell mechanics and the robustness of development networks

performed to explore embryo robustness (Farge 2003). (3) Visualization: The ability to accurately validate perturbation in real-time is essential and can be done via coupled high resolution microscopy and imaging with microfluidic chip (Dagani et al. 2007; Cui et al. 2008). Another essential feature is the ability to extract embryos after perturbation for further analysis of mutations and developmental robustness.

2 Internal perturbation: miniature RNAi technology

Injection of genetic entities into model organisms, such as *Drosophila*, is an internal perturbation method that has been a topic of active research in development biology and genetics. A critical technique in gene silencing, known as RNA interference (RNAi), involves the injection of double-stranded RNA (dsRNA) to silence specific genes (Adams 2000; Agrawal et al. 2003; Buckingham 2004). This powerful experimental technique helps scientists correlate the induced phenotypic changes to specific gene functions. Typically, individual embryos are manually injected using glass micropipette needles, which are a labor intensive, inefficient, and a highly invasive procedure that typically leads to poor control over the injection process and inconsistencies in injection conditions and results. There is increasing demand for precise and controlled injection of drugs, DNA, proteins, enzymes, ions, antibodies, and metabolites in living cells and embryos. Here, we discuss the importance of embryo immobilization on open substrates for accurate injection. In addition, we highlight key microinjection technologies for RNAi experiments.

2.1 Immobilization through self-assembly

To immobilize embryos for injections or microscopic inspection, the conventional method calls for the use of embryo glue (Ashburner et al. 2005). This is obtained by dissolving fragments of adhesive tape into heptane. Slides are then covered with this glue to provide a platform for analysis. This method was developed in order to manually position and orientate individual embryos using a light microscope, but it is time consuming and leaves a high margin of error. It is especially counter-productive given our ever more sophisticated abilities to operate at the micro level, which should allow us the ability to accurately and automatically immobilize and orient embryos in specific arrays for high throughput experiments. New methods will dramatically improve on the shortcomings inherent in the manual placement process, add versatility to the perturbation process, and allow accurate real-time imaging of embryos.

Bernstein et al. (2004a) reviewed three techniques for the positioning of *Drosophila* embryos in 2D arrays. One is aspiration, a simple technique which involves micromaching micro-holes on a substrate and applying suction to immobilize the embryos in arrays (Wang et al. 2007a). The size of the micro-holes and the strength of the aspiration dictate capture efficiency. Wang et al. reported the use of a vacuum-based embryo immobilization device that consisted of an array of through-holes with a diameter of 400 μm connected to a backside negative pressure of 2–7 inHg. Zebrafish embryos were efficiently trapped and injection experiments were successfully performed. Such a device can also be applied for *Drosophila* embryo immobilization. However, there is no control over the embryo orientation and there is a possibility of physical damage to the embryo. A second potential capture method that may be utilized involves dielectrophoresis. This technique is commonly used to immobilize and manipulate individual cells and microorganisms. For example, single cells were successfully trapped and levitated in a microwell using negative dielectrophoresis (Bocchi et al. 2008). A microchannel at the base of the device provided fluid flow through capillarity in the microwell. Consequently, dielectrophoresis can be combined with microfluidics to immobilize *Drosophila* embryos for perturbation. Metal microelectrodes patterned on a substrate can be used to create and control the dielectrophoretic force necessary for accurate embryo placement. However, this technique has not been demonstrated on embryos and the effect of dielectrophoresis on embryo viability has not been reported.

A third technique, fluidic self-assembly (FSA), has emerged as a promising method for automatic immobilization and positioning of embryos on an open substrate (Boringer et al. 2001). As shown in Fig. 3a, FSA uses fluids to deliver micro-scale objects to specifically engineered immobilization sites (Bernstein et al. 2004a, b). Embryos positioned by FSA involves two main steps: surface modification and embryo assembly. Utilizing appropriately engineered surfaces and physical/chemical interactions, such as surface tension, hydrophilic/hydrophobic and electrostatic interactions, is important to create well-defined immobilization sites. In addition, an appropriate carrier fluid, that allows efficient delivery and rapid assembly while maintaining *Drosophila* embryo viability, is essential for the embryo transport step. Embryo assembly is governed by the minimal potential-energy principle which states that an object (embryo) will seek a minimal potential energy state at the assembly site to achieve thermodynamic stability (Dagani et al. 2007). During FSA, a lower interfacial energy between the embryo's surface and the adhesive layer (or oil bridge) on the surface of the immobilization site is achieved. This increases the

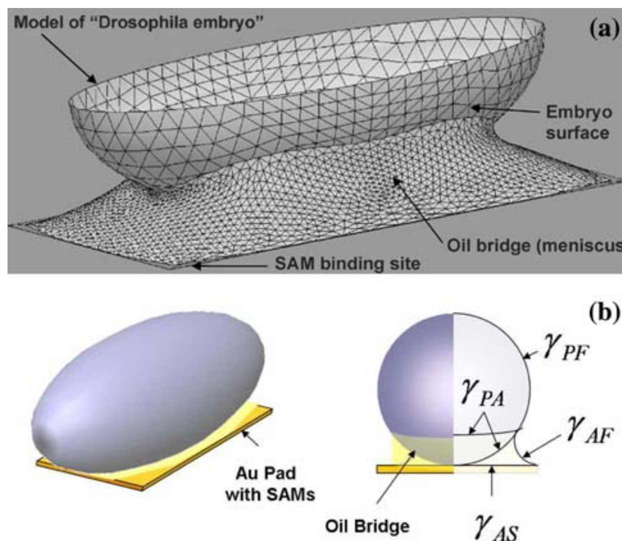


Fig. 3 The concept of fluidic self assembly. Accurate embryo immobilization and orientation is important for high throughput analysis. Fluidic self assembly (FSA) is an automatic and efficient method of positioning embryos on both an open substrate and within a microfluidic channel. **a** FSA is achieved when an embryo reaches thermodynamic stability at an assembly site. An oil adhesive bridge is formed between the self-assembled monolayer (SAM) binding site and the embryo surface (Bernstein et al. 2004a, b). **b** Surface wetting can be used to describe the capillary force between two different surfaces in contact with an adhesive bridge. The interfacial energies γ acting on an assembled embryo are shown between the particle surface (*P*), the liquid adhesive (*A*), the surrounding carrier fluid (*F*), and the substrate surface (*S*). The interfacial energy between the adhesive and the particle should be smaller than the energy between the particle and the carrier fluid. A low interfacial energy between the particle's surface and the adhesive promotes the surface wetting of the particle, and has the potential for pulling the particle into the energy well for assembly (Dagani et al. 2007)

capillary forces between the two surfaces, and thus, attracts the embryo onto the assembly site (Fig. 3b).

Self-assembly of *Drosophila* embryos were demonstrated, as shown in Fig. 4a, using oil covered self-assembled monolayers (SAMs) patterned on an open silicon substrate (Bernstein et al. 2004b; Zhang et al. 2005). The SAMs consisted of thiol-treated gold pads which rendered them hydrophobic and allowed selective localization of halocarbon oil. Accordingly, de-chorionated *Drosophila* embryos, dispensed on the surface of the substrate using water, selectively adhered to the oil and assembled (Fig. 4a). This is because that the vitelline membrane, revealed after removal of the chorion, is hydrophobic. The interaction between the vitelline membrane and the oil-covered SAMs ensures successful assembly while maintaining embryo viability (Limbourg and Zalokar 1973). Bernstein et al. reported an immobilization yield of 85% on arrays of patterned 250 μm × 400 μm rectangular gold pads but also noticed embryo clustering on individual pads. Further reduction of the pad size to 200 μm × 200 μm reduced the clustering problem. Additionally, it was demonstrated that an average of 40% of immobilized embryos aligned within ±9° along the axis of symmetry of the pad (Bernstein et al. 2004b) which is due to the effects of a restoring torque by the halocarbon oil (Dagani et al. 2007). Consequently, this immobilization method provided an added ability to control the orientation of embryos for high throughput assays.

2.2 Automated MEMS-based microinjection of *Drosophila* embryos

Two powerful methods have been established in the past for such analysis in *Drosophila*: permanent genetic transformation with transposable genetic elements and transient specific gene silencing through RNA interference (RNAi). Both methods require reliable and rapid injection of DNA and dsRNA, respectively, at the earliest stages of embryonic development. Development of robust technologies beyond proof of principle will enable their dissemination and widespread use within the *Drosophila* research community. Application of these new tools will lead to a better understanding of molecular mechanisms of development and disease in humans, with expected significant impact on new therapies and improvement of the state of public health.

Ideally, a high throughput injection screen (Fig. 5) would involve arrays of miniaturized injectors delivering small molecules in parallel to hundreds of well positioned embryos (Bernstein et al. 2004a). The system would be able to inject accurate volumes with controllable penetration forces at a specific location on the embryo membrane. Surface micromachined hollow silicon nitride needles were designed and characterized specifically for injection of DNA and proteins into fruit fly embryos (Figs. 6a, 7). This technology allows precise control over the shape and size of the microinjectors, and accurate calibration of the injected volumes is made possible by using integrated pressure sensors. The capability to do more precisely calibrated and less damaging embryo injection will considerably raise the level of quantitative studies and lead to better understanding of development and cell differentiation in fruit flies (Zhang et al. 2004; Zappe et al. 2006).

The ability to control the penetration force is critical when performing high throughput injections on the embryos. It was reported that a piezoelectric vibratory microinjector for genome-wide RNAi screens on *Drosophila* embryos minimizes the penetration force (Zhang et al. 2006). The MEMS-based injectors consisted of a surface micromachined silicon-nitride probe with an integrated force sensor (Fig. 6a). The optical force sensor

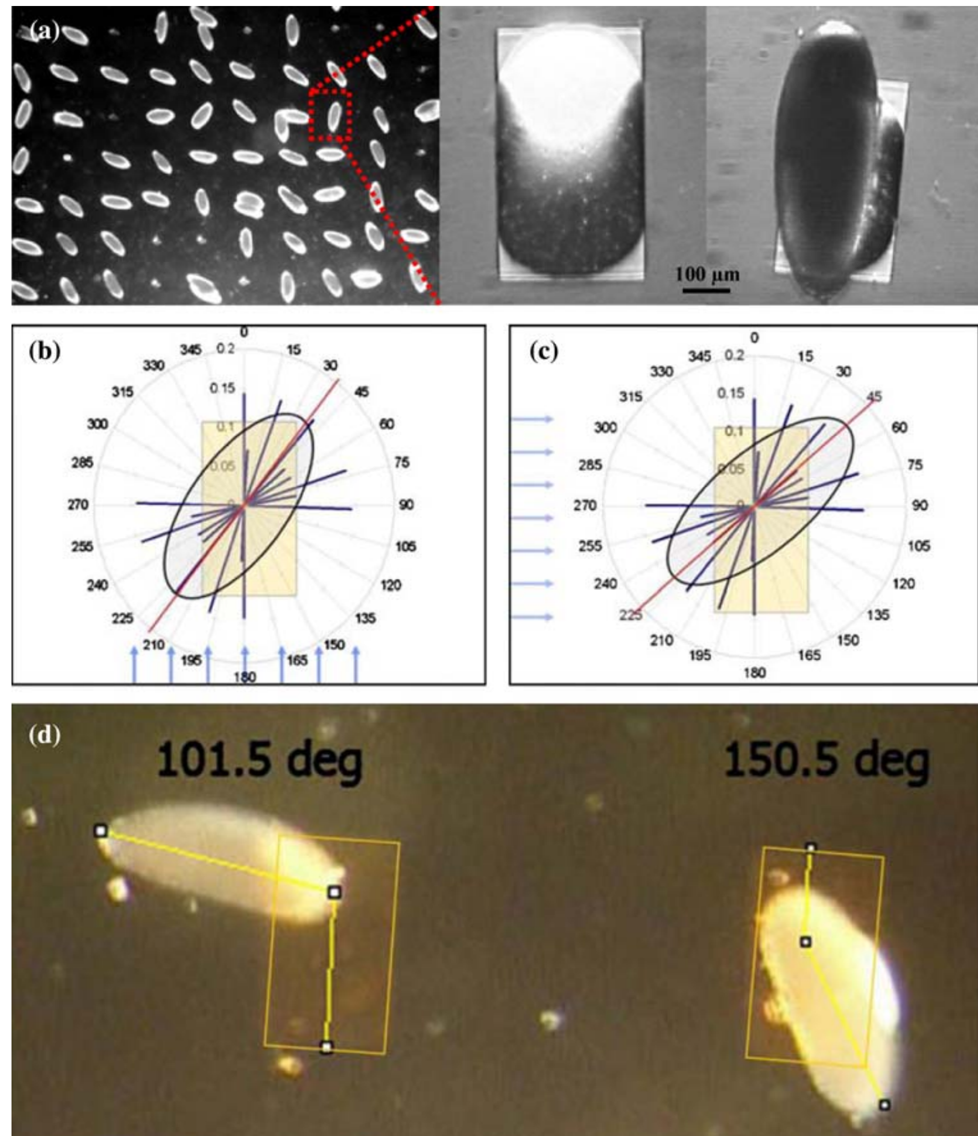


Fig. 4 Fluidic self assembly. (**a**) FSA allows for the automatic immobilization of several embryos in ordered arrays (Zhang et al. 2006). A self-assembled monolayer (SAM) is composed of a gold pad with an oil drop. Embryos are immobilized by FSA on an oil covered SAM (Bernstein et al. 2004b). Radar graphs of the normalized orientation data illustrating the behavior of embryos assembled on pads inline (**b**) and perpendicular (**c**) to flow conditions. The longest *line*, bisecting the embryo outline, indicates the average orientations for each set of data. Embryos assembled onto pads inline with flow averaged a smaller orientation offset (36°) than those assembled onto pads perpendicularly oriented to flow (46°). The *arrows* indicate flow direction with respect to the outline of the Au pad. (**d**) Two embryos are assembled onto rectangular pads and their orientations are measured using ImageJ software (Dagani et al. 2007)

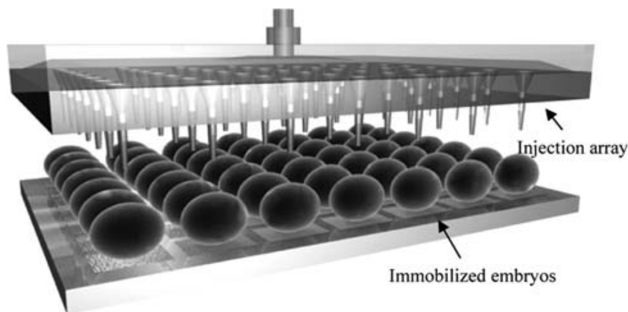
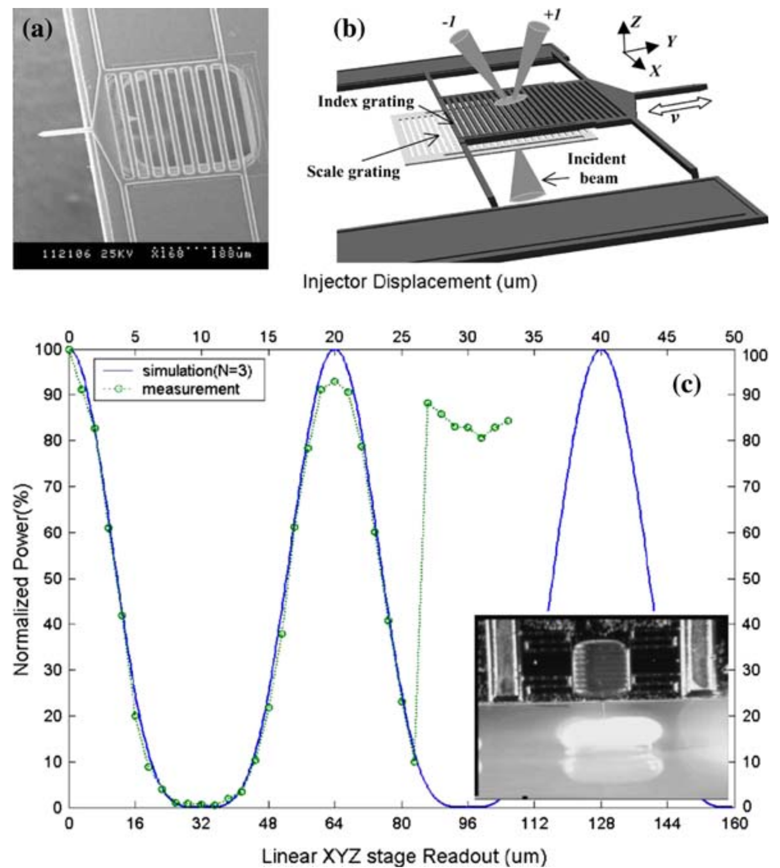


Fig. 5 An ideal high throughput microinjection system (Bernstein et al. 2004a)

consists of two perfectly aligned but separated micro-gratings (Fig. 6b). The index grating is connected to the micro-probe and is suspended by four springs of known spring constants. The scale grating is a fixed grating that is used to measure displacement which is then translated to a measure of the penetration force at the injector tip (Fig. 6c). To reduce the penetration force, the microinjector device was ultrasonically agitated by a piezoelectric actuator. The combination of the MEMS-based probe with the optical sensor allows for closed loop control of actuation. As a result, this setup permitted a shorter probe penetration distance, a faster injection rate, and minimal membrane damage. Injections performed using a conventional glass micropipette and the microinjector were compared. The penetration force on a *Drosophila* embryo using the microinjector with a 30 µm probe tip was approximately a fourth of the force produced when using a glass needle with a diameter of ~75 µm. As a result, a reduced penetration force due to a smaller tip size and ultrasonic vibration significantly reduced the damage and leakage from the outer membrane of the embryo. In addition, due to the ability to

Fig. 6 MEMS-based injector with integrated force sensor for injecting an immobilized embryo. **a** Scanning electron micrograph of the MEMS-based injector. **b** The force encoder is composed of a fixed scale grating and the index grating attached to the microneedle. The force sensor measures microinjector displacement and penetration force. **c** The force sensing of the MEMS-based injector has a large dynamic range. The graph was measured with a laser spot size of 60 μm ($N = 3$), a half-grating pitch period $L = 10$ mm, and the spring constant of the index grating structure Ky = 1.85 N/m. The measured embryo injection force was $F = 48$ μN. The inset shows the operation of the MEMS-based injector (Zhang et al. 2006)



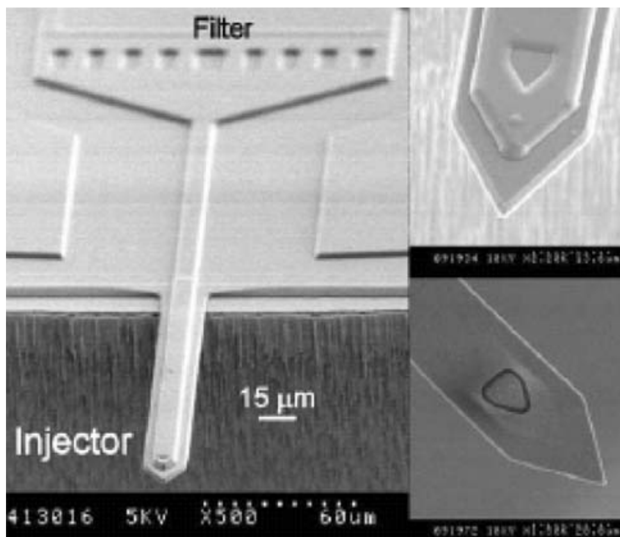


Fig. 7 SEMs of MEMS-based injectors (Zappe et al. 2006)

monitor the penetration of the microinjector, there is better control over the injection process. For example, the microinjector penetration depth can be precisely and mechanically controlled, in contrast to manual injection, which depended on the skill of the trained worker using a glass needle. Manual injection is time consuming and several embryos may be wasted due to double penetration (penetration through one side of an embryo and exit through the other side) or inaccurate injection. Consequently, it was reported that the microinjector successfully injected 100 embryos in less than 7 min using a piezoresistive pressure sensor to control the injected volume. The integration of this configurable and highly sensitive microinjection system with FSA to accurately position embryos will result in a powerful research tool for high throughput injections. The research will advance fundamental engineering knowledge regarding design, fabrication, packaging, and application of MEMS devices for embryo microinjections. The generated knowledge can help with the development of novel systems for automated handling of DNA, RNA, biochemical reagents, cells, oocytes, embryos, as well as micro- and nanoparticles, with widespread applications in biological research, biotechnology, drug discovery, high-throughput screening, and medical diagnostics.

2.3 Automated RNA interference

The development of highly reproducible, fully automated microinjection systems that leverage image acquisition; processing tools with microrobotic control; and embryo immobilization techniques is critical to enabling and

exploiting high throughput injection of *Drosophila* embryos. The MEMS-based injector system, developed by Zappe et al. scans the surface of a glass slide for embryos and performs precise injections for high throughput RNAi screens. An embryo detection setup, which consisted of two CCD cameras controlled by a LabVIEW program, was used to scan for embryos and facilitate the injection process to precisely position delivery and to avoid double injection of embryos (Zappe et al. 2006). The MEMS-based microinjector chip, which consists of a hollow silicon nitride needle coupled with a Pyrex microfluidic system for dispensing of DNA and RNA solutions (Fig. 7), along with embryos, was mounted on motorized xyz-stages. The system automatically screened for embryos and injected a typical volume of 60 pl of dsDNA solution per embryo. The automated microinjector system successfully performed an RNAi experiment using dsRNA corresponding to the segment polarity gene *armadillo* (Fig. 8a, b). It reliably injected 98% of all embryos on the glass slide and almost 80% expressed a loss-of-function phenotype. Integrating this system with an automated confocal microscopy system would result in a powerful tool for high throughput RNAi screens with efficient phenotype analysis capabilities. The automated microinjection system, designed by Zappe et al., is limited to vertical injections because the MEMS-based microinjector chip is positioned vertically above the embryo and penetrates downwards into its side. However, transgenic fruit flies are produced by injecting DNA into the posterior tip of the embryo. Recently, Cornell et al. developed an automated microinjector system that injects embryos horizontally (Cornell et al. 2008). This allows for the ability to penetrate an embryo at the poles as well as on the sides (Fig. 9). The system consisted of an inverted microscope with a motorized xy stage, a CCD camera and an injection needle. It was controlled using software development in C# language and MySQL was used for data management. In addition, an alignment jig, which consisted of an array of well designed grooves on a stainless steel block, was used to align embryos on a coverslip. The alignment jig, however, lacks the flexibility to pre-screening the embryos in vivo to ensure that they are at the right stage in development. In addition, the adjustment of embryo orientations is limited comparing to that of a self-assembly pad (Cornell et al. 2008). Both drawbacks can be overcome by staging the development of embryos properly prior to the injection process and modifying the system to inject at both ends of the embryo. The overall efficiency of injection is measured by the percentage of transformed embryos. After optimization, it was found that the overall injection efficiency using the robotic system was 50–75% that of manual injection with a rate of 60 embryos in 8 min (Cornell et al. 2008). Although the injection speed and efficiency is comparable to manual injection, further optimization of this automated embryo

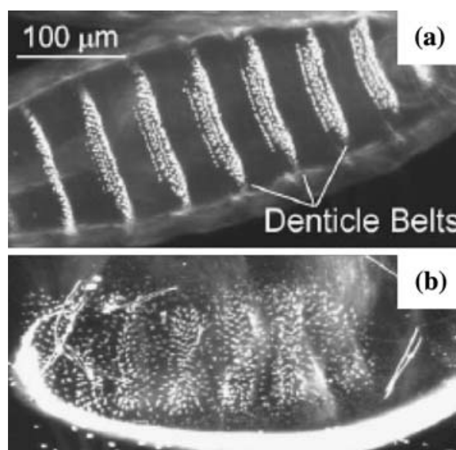


Fig. 8 An automated *Drosophila* embryo microinjection system for RNAi screens. The microinjection system was used to successfully perform an RNAi experiment using dsRNA corresponding to the segment polarity gene *armadillo*. **a** Analysis of the cuticle of a wildtype embryo shows distinct ordered denticle belts, which result in specific segments that later correspond to a part in the adult fly. **b** Injected embryos revealed the complete destruction of the ordered pattern of the denticle belts (Zappe et al. 2006)

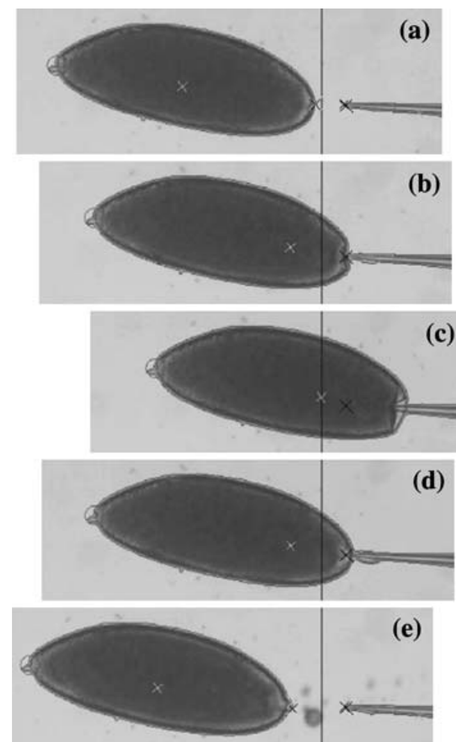


Fig. 9 An automated *Drosophila* embryo microinjection system for transgenic studies. An automated microinjection system was designed to horizontally inject embryos at the posterior end to produce transgenic fruit flies. The system successfully injected embryos at a rate of 60 embryos in 8 min. The image sequence shows an injection process using the automated system (Cornell et al. 2008)

microinjection system is warranted to achieve injections with higher throughput and increased efficiency.

3 External perturbation: micro-environmental perturbation of *Drosophila* embryos

Perturbing the microenvironment is a complementary approach to perturbing the genetic components of the development network (Takayama et al. 2001; Sia and Whitesides 2003; Takayama et al. 2003; Lucchetta et al. 2005). Cell culture techniques in microenvironments have been extensively explored using microfabrication techniques and surface chemistry modifications (Chen et al. 2005). For example, neuron cells were successfully cultured on SAMs to study cell adhesion, interaction and stimulation (Palyvoda et al. 2007). As a result, neuron cells can be cultured on patterned microelectrodes which can potentially facilitate the integration of cells with computational devices to develop neuronal implantable microsystems. Alternatively, a microfluidic device was presented where single HeLa cells were trapped and cultured on arrays of hydrodynamic U-shaped polydimethylsiloxane (PDMS) trapping structures (Di Carlo et al. 2006). Such a device could potentially facilitate high throughput single cell analysis of drug toxicity.

Recently, there has been a growing interest in investigating living organisms at the whole animal level, as it has become clear that the degree of the complexity of biochemical networks may require analyzing the response of the whole system to a perturbation of one component (Breslauer et al. 2006). Revealing the dynamics of biochemical interactions in whole-organisms will provide insight on functional properties, such as developmental robustness and morphogenesis. Living embryos are model systems for such analyses. In the last decade, researchers have used microfluidics technology to culture and probe living embryos. One of the first reports involved using microfluidics for handling and culturing individual mouse embryos (Glasgow et al. 2001). In particular, soft lithography based microfluidics has opened new avenues for embryo analysis in microenvironments because of two major capabilities: (1) the ability to functionalize and precisely pattern surfaces that are biologically compatible, and (2) the versatility to rapidly fabricate application-specific microfluidic systems at sizes relevant to embryos. Microfluidic channels are a favorable interface for studying *Drosophila* embryos because of its flexible size dimension, transparent polymeric structure for live imaging, and ability to deliver precise physical and chemical cues using laminar flows. Because of the extensive experimental permutations required to study genetics holistically (Carpenter and Sabatini 2004), it is beneficial to incorporate all aspects of embryo manipulation into the device. Such handling includes the introduction, immobilization, precise microinjection, environmental perturbation, and collection of embryos in bulk. In addition to environmental control, microfluidics is capable of sample preparation and experimental sorting. The advancement of microfluidic platforms has opened doors to embryo micro-total analysis systems which incorporate instruments for probing and detection.

3.1 Immobilization within a microfluidic channel

We extended the FSA method by incorporating SAMs in microfluidic channels, as shown in Fig. 4d (Dagani et al. 2007). Surface Evolver simulations were used to analyze the surface energies and rotational displacements of assembled embryos on various pad geometries. Embryo orientations on pads after microfluidic self assembly were also verified experimentally (Fig. 4b–d). In addition to improved automatic immobilization, microfluidic self assembly (μFSA) has two advantages: (1) the capability of precise embryo transport using laminar flow, and (2) the ability to detach embryos for further investigations after perturbation. Embryo detachment is attained when an embryo experiences an external force that disrupts assembly stability and results in a gain of potential energy. Detachment of assembled embryos in the microfluidic channel was achieved by reducing the environmental interfacial tension between the embryo and oil using an alcohol buffer. Automatic self assembly of embryos within a microfludic channel has facilitated external microenvironmental perturbation through fluid flow. μFSA was used to thermally perturb live *Drosophila* embryos, and to perform drug screens (chemical perturbation).

3.2 Microfluidic thermal perturbation of *Drosophila* embryos

Recently, Lucchetta et al. used a Y-shaped PDMS microfluidic channel to study embryonic developmental compensatory mechanisms in response to external perturbations with high spatiotemporal resolution (Lucchetta et al. 2005, 2006). Following manual placement in the microchannel, the robustness of *Drosophila* embryos was tested by exposing the anterior and posterior regions of a single embryo to an extreme temperature gradient by means of two laminar flows. Using fluorescent immunostaining, the nuclear density was shown to be higher and the Even-skipped gene (gene that codes for embryonic segmentation in early development) expression was faster in the warm region (Lucchetta et al. 2005). Additionally, using histochemical immunostaining, they observed the formation of Paired stripes (formed from Paired protein

expression after Even-skipped) developing first in the warmer half. Despite this change in thermal distribution, the spatial precision of the Even-skipped and Paired stripes remained consistent further into the development of the embryo. This suggests that compensatory biochemical mechanisms account for the developmental robustness of the *Drosophila* embryo to extreme thermal perturbation (Lucchetta et al. 2006).

The microfluidic device used by Lucchetta et al. has great potential for use in the study of embryo development. However, its versatility can be further improved to perform high throughput perturbation on multiple embryos simultaneously with accurate spatiotemporal control. The employment of microfluidics to perturb multiple *Drosophila* embryos can be further developed by streamlining the manipulation, sorting, and positioning of embryos for assay. Performing similar experiments on multiple embryos is instrumental to improving techniques for genome-wide screening, and automating this procedure will reduce drawbacks of manual embryo placement such as potentially misaligning the embryo in the channel and incurring a large overhead in setup time. The alignment or misalignment of the embryos is crucial for perturbation at the micro-scale, because it skews the flow profile and reduces the perturbation areas by widening the thermally neutral zone in the channel center (Lucchetta et al. 2006).

We introduced an in-channel immobilization technique that will provide enhanced spatial and temporal control over parts of a developing *Drosophila* embryo (Dagani et al. 2007). The device was used to examine early development, particularly cellularization which is characterized by the ingression of the furrow front. The automation of *Drosophila* placement inside a PDMS microchannel was achieved via microfluidic self-assembly (μFSA) using an adhesive oil pad patterned on glass (Fig. 10a). Thermal perturbation was performed using the setup depicted in Fig. 10b. Cellular events of assembled embryos were recorded in real-time using differential-interference contrast (DIC) microscopy (Fig. 10c). A binary thermal flow exposing the anterior of an embryo to 20°C and the posterior to 35°C revealed asynchronous advancement of the furrow front with faster progression on the warmer region (Fig. 11). Following thermal perturbation, embryos were detached using alcohol, which

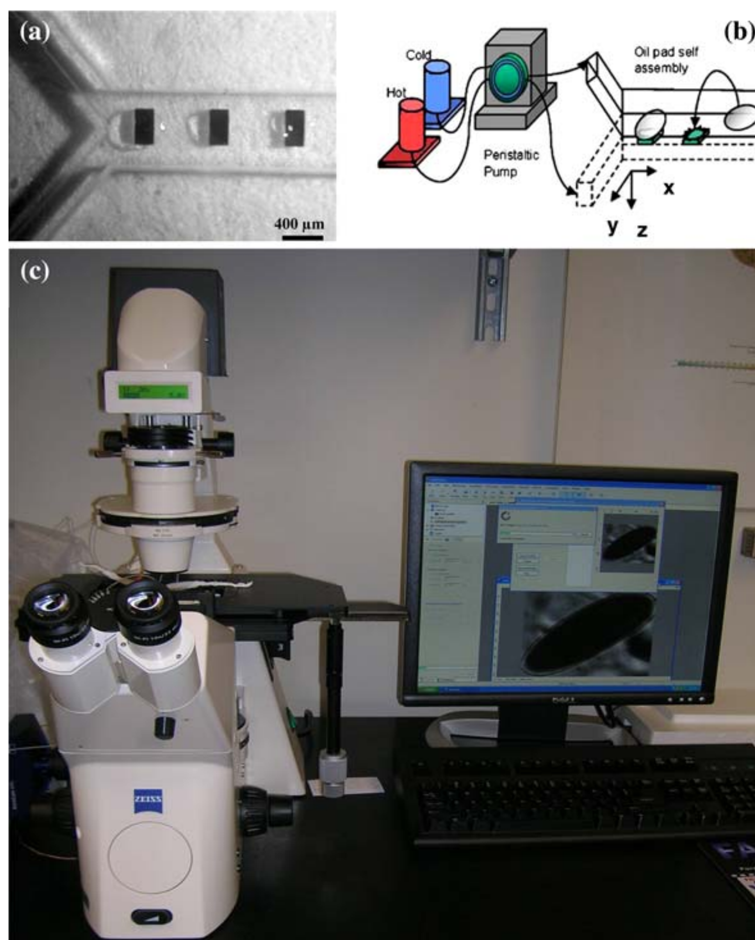


Fig. 10 Microfluidic thermal and chemical perturbation of self assembled *Drosophila* embryos. **a** SAMs embedded in a microfluidic channel. **b** A schematic of the setup used to perform thermal perturbation of assembled embryos inside a microchannel. **c** A time-lapse differential interference contrast (DIC) microscope will allow real-time visualization of thermal and drug effects on developing embryos (Dagani et al. 2007)

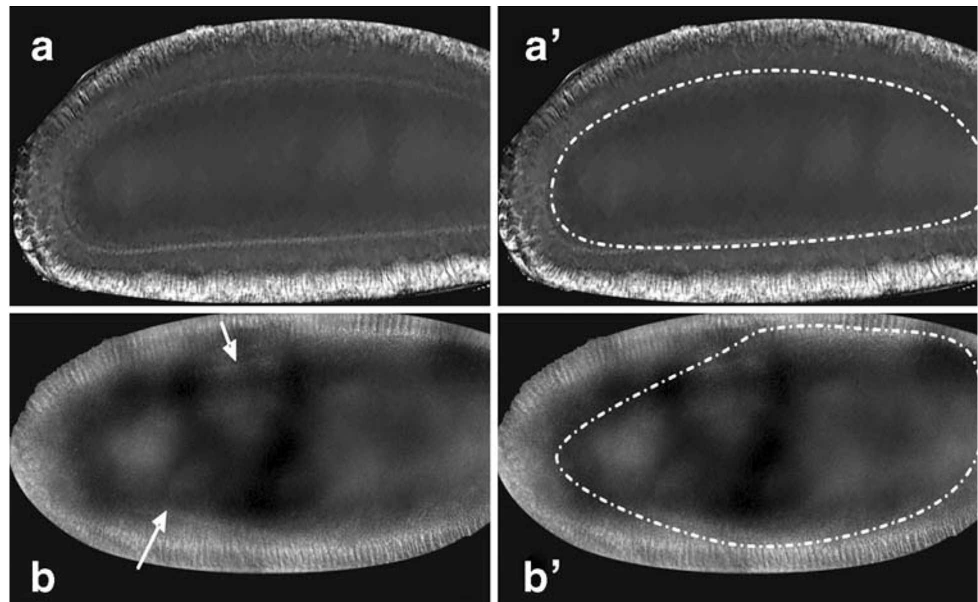


Fig. 11 Microfluidic thermal perturbation results of *Drosophila* embryos. Thermal perturbation revealed asynchronous advancement of the furrow front during cellularization. **a** *Drosophila* embryo undergoing cellularization at 24°C. **a′** Image **a** with the furrow front outlined with a *dotted white line*. **b** *Drosophila* embryo undergoing cellularization while exposed to a thermal gradient. Posterior (*right-side*) is warm (30°C) and the anterior (*left-side*) is cold (20°C). **b′** Image **b** with the furrow front outlined with a *dotted white line*

permitted the device to be reused. Further investigation of developing embryos following perturbation could be performed.

3.3 High throughput chemical perturbation of *Drosophila* embryos

High-throughput drug screens can rapidly identify small molecules (less than 500 Da) that specifically alter biochemical processes in living organisms. We used *Drosophila melanogaster* embryos to observe the primary effects of such drugs on embryogenesis. We developed an assay in which live cellularizing embryos are subjected to a high-throughput screen utilizing 96-well plates. Abnormal morphogenetic movements caused by the effects of potential drugs on RNA metabolism, microtubules, microfilaments, and other cellular components are observable within minutes. These effects were captured through differential interference contrast (DIC) microscopy in the form of still images and time-lapse movies. Once a potential drug is found, its effects could be further characterized in a secondary high resolution screen using a microfluidic device. The microfluidic device provides a higher resolution, optically clear platform on which the primary effects of the positively testing drug can be observed on a single embryo. Here, we show a proof of principle study with data collected from our control drugs, colchicine (399.4 Da) and cytochalasin D (507.62 Da), two compounds known to affect cellularization by specifically disrupting the microtubule and microfilament cytoskeletal systems, respectively (Zhu et al. 2001).

De-chorionated embryos (i.e., embryos whose chorion has been dissolved in bleach solution) are not permeable, and the immediate effects of drug exposure may not be seen without exposure to octane. Octane permeabilizes the embryo by solubilizing the vitelline membrane, which allows diffusion of the drug into the egg (Mazur et al. 1992). Two drugs were tested using the 96-well assay system. Colchicine was selected as a control drug in this assay because it is a well-characterized molecule that binds tubulin and prevents further microtubule polymerization. Furrow formation is therefore inhibited because necessary cell membrane components are not trafficked to the cortex. Cytochalasin D was also chosen because it is well-characterized as a small molecule that caps actin microfilament plus-ends. The membrane furrow fails to form in this case due to the actin (which held the nucleus in position) has been disrupted, and the nuclei fall back into the yolk of the embryo. The high throughput drug screen is divided into two steps: (1) a primary HTS using a 96-microtiter plate, and (2) a secondary screen using a microfluidic device.

3.3.1 Primary HTS using a 96-well plate

Wild type (Oregon-R) embryos were collected and aged at 25°C and processed for drug exposure and imaging. Embryos were de-chorionated in 50% bleach solution for 2 min. Each incubation well contained 50 μL of the drug in 1× phosphate-buffered saline (PBS) containing 1% dimethyl sulfoxide (DMSO) and 50 μL of 99% octane (reagent grade). The embryos lie along the interface of the aqueous and organic layers. The number of embryos in each stage was counted at the beginning of the observation period. The embryos are observed for approximately 30 min with a Zeiss DIC microscope. They were counted again to determine what percentage cellularized. Cellularization

was studied in embryos exposed to solvent alone (PBS+ 1% DMSO with no drug), 150 μM colchicine and 25 μM cytochalasin D. Those exposed to only solvent underwent normal cellularization as expected within the observation period. Of the embryos that were soaked in a 150 μM colchicine solution, only 2% continued to cellularize after 30 min of drug exposure. Those soaked in 25 μM cytochalasin did not cellularize and, in the case, of those embryos in the very early stages of cellularization, actually reversed their cellularization.

3.3.2 Secondary screen using a microfluidic device

The secondary screen involves the use of a microfluidic device that automatically positions live embryos within a fluid-filled imaging microchannel and exposes embryos to defined drug concentrations for fixed periods of time (Fig. 10a, b). While positioned in the microfluidic device live embryos were imaged in real-time by time-lapse DIC microscopy allowing us to assess primary effects on embryogenesis (Fig. 10c). The microchannel was designed to have dual-inlet geometry. This geometry was chosen to expose an embryo to either a single or binary flow of drug solutions. The embryos were collected, aged, and de-chorionated as in the primary screen. They were then soaked in 99% octane for 1 min to be permeabilized. Following a thorough rinse with embryo wash buffer, an embryo was assembled into the PDMS microfluidic channel. A syringe pump (Harvard PHD 22/2000, Harvard Apparatus) was then used to deliver drug solutions at a fixed flow rate of 1 ml/min. Representative movies and still images were acquired using the DIC microscope for a 30 min drug exposure experiment. The effect of each drug was experimentally verified on three embryos each during three separate exposures. Initial control results of embryos exposed to a single flow of 150 μM colchicine or 25 μM cytochalasin D within the microfluidic channel were consistent with results from the 96 well primary screens, as shown in Fig. 12.

Using the microfluidic channels in the secondary drug screening enables the close coupling between microchip-based manipulation and real time high resolution imaging using microscopes. In addition, it allows more accurate temporal control over drug flow. For example, assembled embryos in the microfluidic channel can be exposed to two distinct drug solution flows in parallel or in series. Therefore, an embryo can be exposed to two drugs simultaneously using binary laminar flow. Or, an embryo may be exposed to one drug for a fixed period of time before switching to another solution. The effects of drug exposures in parallel using binary flow may yield completely different results on embryo development than does drug exposure in series. Such extreme microenvironmental perturbations could be used to test the effects of different drug combinations, and furthermore, they could lead to novel therapeutic discoveries.

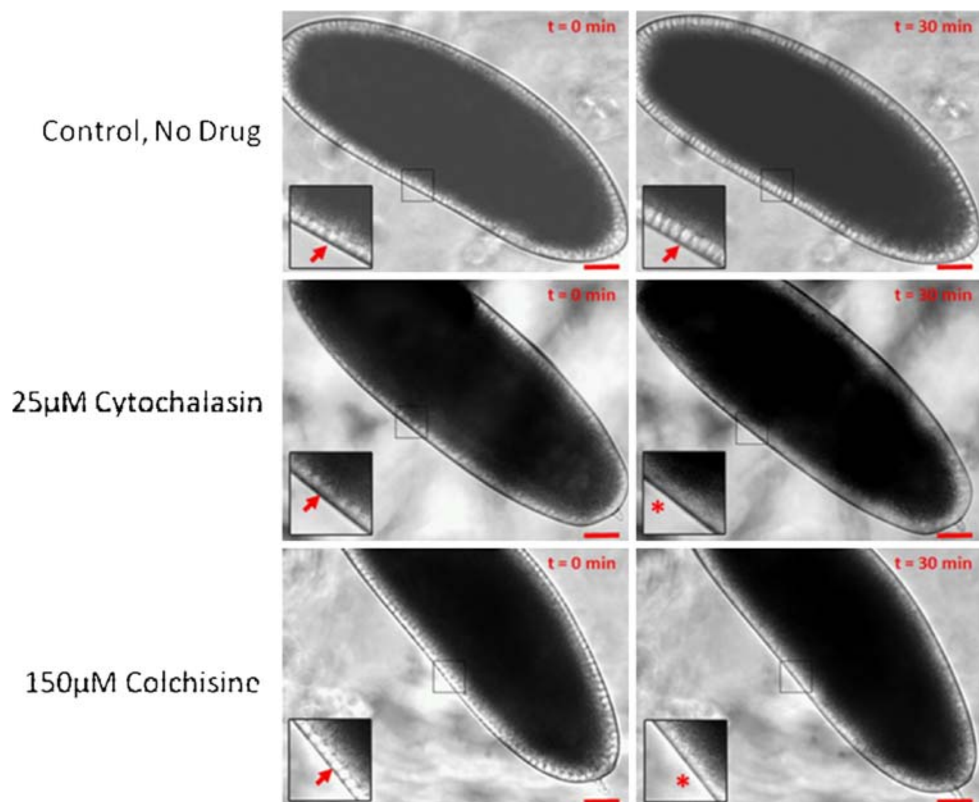


Fig. 12 High resolution effects of 25 μM cytochalasin D and 150 μM colchicine on cellularizing embryos in a microfluidic channel. Imaging using the DIC microscope was started during nuclear cycle 13 for duration of 30 min. The inset is a blow up of the black square on the surface of each embryo. An *arrow* indicates a specific nucleus. An *asterisk* indicates that there are no visible nuclei. The results are comparable to that obtained in the primary screen. The *scale bars* shown here is 50 μm

Wild type and various GFP transgenic *Drosophila* cleavage stage embryos are well suited for high-throughput screening of small molecules that inhibit important cellular processes. First, and foremost, they are robust embryos of normal ploidy and developmental potential. Therefore, we expect that relatively little false positive identification will be made due to system weaknesses. In addition, they can tolerate incubation in 1× PBS/1% DMSO and octane without drugs for at least 30 min without measurable effects on morphogenesis. Second, they can be collected in large numbers inexpensively (Sisson 2000). Third, we know that several drugs that disrupt specific processes in human cells also disrupt these processes in *Drosophila* cleavage stage embryos upon injection, resulting in characteristic phenotypes that are readily identified in 96-well microtiter plates by DIC or fluorescence microscopy. These drugs include the DNA synthesis inhibitors actinomycin D and aphidicolin (Zalokar and Erk 1976; Beebe et al. 2000), the mRNA synthesis inhibitor α-amanitin (Edgar et al. 1986), the protein synthesis inhibitors cycloheximide, puromycin, and pactamycin (Zalokar and Erk 1976; Edgar and Schubiger 1986), the microtubule depolymerizing drugs colcemid and colchicine (Zalokar and Erk 1976; Edgar et al. 1994; Sisson et al. 2000; Zhu et al. 2001), the microfilament depolymerizing drugs cytochalasin B and D (Zalokar and Erk 1976; Sisson et al. 2000; Zhu et al. 2001), the membrane vesicle transport inhibitor brefeldin A (Sisson et al. 2000), and the RhoGTPase inhibitor *Clostridium botulinum* C3 exoenzyme (Ali and Crawford 2002). Fourth, once new inhibitory drugs are identified, the excellent biochemistry offered by cleavage stage embryos (Sisson 2000) affords the opportunity to identify the endogenous target molecule(s) using affinity purification techniques. Discovery of drugs that affect development in flies may lead to the elucidation of the pathways of disease genes. It is a small, but important, stepping stone between studying mechanisms of action in single cells (i.e., yeast and slime mold) and disease models in vertebrates (i.e., mice).

4 Conclusion

The advantages of using *Drosophila* as a model system for establishing the genetic basis of cell growth and differentiation in humans is well established and recognized with three Nobel Prizes in Physiology and Medicine. *Drosophila* allows in vivo genome-wide and high-throughput screening using advanced microtechnologies due to its ease of manipulation, amenability to imaging, and ability to grow in minute volumes. To date, however, the methods to conduct such screens have not been fully optimized. Even simple assays at relatively large-scales still take months to years to complete. In addition, due to the lack of key technological integration, several assays either cannot be performed at all or have to be dramatically simplified for high-throughput screens. In this paper, we have highlighted recent progress on the development of miniature tools to quantify critical cellular processes in *Drosophila* development, through investigating the impact of genetic variation and microenvironmental perturbation on intra-cellular biochemical signaling pathways, cell mechanics and the robustness of development networks. These technologies will not only significantly improve current automated screening methods but also open doors to design adaptive, robust, reproducible tools amenable to the large-scale investigation of the relationship between precise perturbation and embryo biochemical and functional responses. These microsystems technologies and high-throughput assay strategies can significantly impact discovery of new drugs that target molecular mechanisms involved in human genetic disorders.

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