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Laser direct-write of single microbeads into spatially-ordered patterns

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Abstract
Fabrication of heterogeneous microbead patterns on a bead-by-bead basis promotes new opportunities for sensors, lab-on-a-chip technology and cell-culturing systems within the context of customizable constructs. Laser direct-write (LDW) was utilized to target and deposit solid polystyrene and stem cell-laden alginate hydrogel beads into computer-programmed patterns. We successfully demonstrated single-bead printing resolution and fabricated spatially-ordered patterns of microbeads. The probability of successful microbead transfer from the ribbon surface increased from 0 to 80% with decreasing diameter of 600 to 45 μm, respectively. Direct-written microbeads retained spatial pattern registry, even after 10 min of ultrasonication treatment. SEM imaging confirmed immobilization of microbeads. Viability of cells encapsulated in transferred hydrogel microbeads achieved 37 ± 11% immediately after the transfer process, whereas randomly-patterned pipetted control beads achieved a viability of 51 ± 25%. Individual placement of >10 μm diameter microbeads onto planar surfaces has previously been unattainable. We have demonstrated LDW as a valuable tool for the patterning of single, micrometer-diameter beads into spatially-ordered patterns.

(Some figures may appear in colour only in the online journal)

Introduction

The precise placement of physiologically relevant microbead subunits into spatially-ordered patterns has broad implications for biomaterial processing and biomedical applications. Microbeads possess versatile applicability as probes for biomolecule screening [1], vehicles for targeted drug delivery [2, 3], microtools for cell manipulation [4] and microbioreactors for scalable cell culture systems [5, 6]. For example, positioning of cell-laden or protein-conjugated beads from blueprinted patterns can greatly enhance spatial detection in bioMEMS sensor technology [7–9], and the arrangement of drug-loaded microbeads allows for the formation of spatially defined concentration gradients [10]. In addition, recent work has made it possible to encapsulate cells in physiologically relevant biomaterials capable of directing biological processes [11, 12]. Hydrogel beads can function as isolated 3D microbioreactors, analogous to in vivo microenvironments, for cell maintenance and differentiation [6]. Patterning cell-laden microbeads may prove useful as cell-based combinatorial arrays for lab-on-a-chip devices [13] and building blocks for engineered tissue construction [14–16].

Microbead patterning technologies facilitate the placement of solid microspheres or engineered hydrogel
microenvironments into specific positions on substrate areas. The ability to selectively deposit microbeads on a bead-by-bead basis allows researchers the freedom to fabricate custom patterns, with the benefits of bottom-up engineering (i.e., the generation of large constructs by assembly of smaller, repeating functional subunits). Both solid and hydrogel microbeads are attractive subunits for bottom-up engineering because of their well-defined shape, tunable mechanical properties, definable surface chemistry and biomimetic 3D microenvironment. In addition, microbead biochemical properties (e.g., composition, presentation of ligands or receptors, encapsulated cell density) can be customized without affecting the overall printing processes. The ability to localize functional or bioactive subunits into virtually any configuration broadens the applicability of microbead patterns for biomedical devices and engineered tissue constructs. Particle selection and positioning according to their physical size defines the sensitivity and resolution of actuation in many of the applications. Thus, bottom-up printing techniques are ideal for size-selective positioning of microbead subunits into spatially-ordered patterns.

Current technologies for patterning microbeads include template-assisted assembly, dip-pen lithography, optical laser trapping and laser direct-write (LDW). Template-assisted assembly is a top-down technique that utilizes textured surfaces with differential topographical heights for selective microbead entrapment and adsorption onto an underlying, planar substrate surface [17–20]. Dip-pen lithography modifies the atomic force microscopy tip for the deposition of microbead suspensions by resist-write mechanisms [21]. However, by the inherent design of these dispensing techniques, the ability to select a desired microbead from a suspension and pattern the targeted bead into a chosen position is not possible. To address this problem, optical laser trapping technology was developed for the manipulation of individual spherical objects [22–24]. However, size restrictions for effective particle movement limit the ability to scale-up patterns [25].

In this study, we demonstrate and characterize the feasibility of LDW for the systematic deposition of microbeads into spatially-ordered patterns, with single-microbead resolution. Direct-writing refers to the deliberate assembly of computer-aided manufacturing (CAD/CAM) and camera-equipped technique that has been shown to precisely deposit and pattern nano-biomaterials [26], proteins [27–30], bacteria [31], mammalian cells [32–34] and embryonic stem cells [35]. LDW accurately, precisely and quickly deposits voxels (volume pixels) of biomaterials with high spatial resolution (±5 μm) for fabrication of biological constructs and patterns [32]. An advantage of LDW is the ability to pattern specific objects by camera-aided targeting and planar control of substrate positions defined by computer-aided designs [12]. LDW addresses all of the requirements for ideal single-microbead deposition into spatially-ordered patterns. In this technique, excimer laser–material interactions propel camera-targeted microbeads, partially embedded on a print ribbon, onto a receiving substrate, resulting in soft transfer. Through independent ribbon and substrate stage controls, single microbeads are targeted in real time and additively deposited into programmable array positions. When a ribbon is loaded with microbeads of different diameters, larger beads may be transferred by simply increasing the laser beam spot diameter. Additionally, recent efforts demonstrated the feasibility of laser-based transfer of polystyrene microbeads into discrete voxels [36]. We also demonstrate that this process is a gentle technique able to transfer cells encapsulated in hydrogel microbeads. Mouse embryonic stem cells (mESCs) were used as a model cell line due to the sensitivity of both their viability and pluripotency to mechanical stimulation [12, 37]. It has previously been shown that direct-written mESCs were not affected by nanosecond exposure to UV light and maintained their pluripotency [38]. The capability to direct-write alginate microbeads provides exciting new venues and functionality for hydrogel microbeads. By spatially controlling single bead placements, high-resolution heterogeneous constructs from discrete subunits could be fabricated.

Materials and methods

Polystyrene solid microbeads

Divinylbenzene-cross-linked, solid polystyrene microbeads (Polysciences Inc., Warrington, PA) with diameters of 45, 90, 250 and 600 μm were used. Manufacturer’s measured specific gravity was 1.04 and assumed to be isotropic throughout each solid sphere. Monodisperse microbeads were suspended in sterile, deionized water at a mass-equivalent concentration of 0.42% w/v. Suspensions were warmed to 37 °C and titrated to ensure uniform bead distribution before use.

Hydrogel microbeads and cell encapsulation

Porous alginate microbeads were prepared by high-voltage electric field-driven fabrication and coated with poly-L-lysine (PLL) and 0.15% alginate [5]. The bead diameter targeted for direct-write was ∼200 μm. Beads were suspended in 1.5% w/v CaCl2 or Dulbecco’s minimum essential medium (DMEM), and warmed to 37 °C prior to printing. Similarly, trypsinized mESCs were mixed with alginate solution and encapsulated within alginate microbeads. Some beads were also coated with subsequent layers of PLL and alginate. Cell-laden beads were suspended in mESC maintenance medium containing leukemia inhibitory factor (LIF) and maintained in a standard cell-culture incubator (37 °C, 95% RH, 5% CO2) prior to LDW.

Laser direct-write

Microbead patterning was achieved by exploiting excimer laser/material interaction as the printing mechanism [32], as shown in figure 1. A single ArF laser pulse (Teosys, Crofton, MD; near-Gaussian energy distribution, fluence = 0.44 ± 0.07 J cm−2 and pulse width = 8 ns), operating at 193 nm wavelength, locally evaporates the gelatin dynamic release layer (DRL) on the print ribbon, resulting in vapor
pocket formation over the target area. The directed vapor expansion expels the underlying materials of interest as an ejected droplet containing the desired bead onto the receiving substrate. Between each microbead transfer, the substrate stage is translated to the next pattern position in accordance with the g-code blueprint. Adapted from [43].

Figure 1. Schematic of the LDW setup for synchronous positioning and printing of microbeads over desired pattern spots on a substrate. A single laser pulse delivered to the UV-transparent ribbon causes local evaporation of the DRL, where the targeted microbead is partially encapsulated. The resultant vapor pocket ejects a droplet of the receiving substrate. Between each microbead transfer, the substrate stage is translated to the next pattern position in accordance with the g-code blueprint. Adapted from [43].

Visualization of microbeads on ribbon surface

We examined the spatiotemporal positioning of polystyrene microbeads within the gelatin DRL-coated ribbon surfaces. Ribbons were loaded with beads according to the preparation protocol outlined above. As shown in figure 2(A), a USB camera-equipped, compound microscope (Konus) was oriented along the transverse plane of the ribbon, such that the ribbon was situated as it would have been positioned for LDW. Micrographs and videos were captured with the manufacturer-provided program (PhotoImpression, Arcsoft).

Assessment of successful transfers

The probability of successful transfers of single microbeads was measured for polystyrene microbeads with diameters of 45, 90, 250 and 600 μm by counting the number of successful ejection events when laser direct-writing 20 unique beads, each receiving a single laser pulse exposure. The definition of a successful transfer necessitated the deposition of a single, targeted microbead from the ribbon surface to a receiving substrate. Events deemed as unsuccessful transfers included failure of material to eject upon delivery of a laser pulse, deposition of >1 bead per transferred spot or ejection of DRL droplet without a microbead.

Assessment of cell viability

Approximately 5 min after printing, cell viability in LDW hydrogel microbeads was evaluated with live/dead staining (BioVision) \((n = 10\) microbeads). The ‘live’ dye is cell permeable and the ‘dead’ dye is cell impermeable, but accumulates in cells with disrupted plasma membranes. Control beads were pipetted from suspension onto coated substrate surfaces and allowed 5 min to settle into a random ‘pattern’ before the supernatant was removed \((n = 10\).

Adherence tests

Transferred microbeads were immobilized on the substrate for long-term cell culture or post-processing. Immediately following LDW, microbead patterns were immersed in 2% w/v CaCl₂ for 5 min prior to imaging. For long-term immobilization, patterns were immersed in 2% w/v CaCl₂ or
Figure 2. Imaging setup used to capture side-view micrographs of adsorbed microbeads on ribbon surfaces coated with gelatin DRL. (A) A USB camera-equipped microscope was manually aligned parallel to the bead-loaded transverse plane of the ribbon. (B) Bright field (100 ×) images of various diameter beads. The white dashed lines indicate the peripheral surface of the microbead. In images where the microbead-loaded plane did not completely align with the microscope horizontal, reflections appeared on the gelatin DRL, as indicated by the white arrows. Scale bar = 90 μm. (C) Frames from time-lapse video capturing the receding meniscus surrounding a 45 μm diameter bead. The arrow indicates an artifact on the DRL surface. (D) Frames from time-lapse video capturing the receding meniscus about a 600 μm diameter bead.

cell culture media for 24 h prior to further processing. To test the adherence of direct-written microbeads, L-shaped fiduciary markers were laser-micromachined directly onto Petri dish substrates prior to LDW. Direct-written and immobilized microbead patterns in DMEM or 2% w/v CaCl₂ were then incubated for 24 h under standard cell culturing conditions, before they were subjected to disturbances by exposure to an ultrasonic bath (Branson, 3510, Danbury, CT). In the turbulence tests, the substrate containers were partially immersed in 1.5 L volume of 37 °C deionized water for 1, 5 and 10 min. The ultrasonicator generated uniform, ultrasonic waves at a frequency of 40 kHz. Images were taken at each time point with an inverted compound microscope (Carl Zeiss, Thornwood, NY). The distances of each microbead center relative to the location of fiduciary marker intersection were determined with a built-in image analysis program (Axiovision).

Scanning electron microscopy

The surface morphology of printed alginate microbeads and alginate bead–substrate interface were characterized with a field-emission scanning electron microscope (FE-SEM, JEOL JSM-6330F). Microbead patterns were dehydrated for SEM
imaging by serial (10, 30, 50, 70, 90 and 100% v/v in 2% w/v CaCl₂) dilution in ethanol over a 48 h period, prior to sputter-coating with a Au/Pd (60/40) target.

Results

Adsorption of microbead on a ribbon surface

We examined the influence of microbead size on bead adsorption onto gelatin-coated ribbon surfaces using polystyrene microbeads of various diameters (45–600 μm). The imaging setup in figure 2(A) captured the events occurring at the edge of the ribbon–microbead interface, at ambient temperature and relative humidity of ~50%. In each micrograph in figure 2(B), a water meniscus extending from the ribbon surface to the peripheral bead surface facilitated adsorption of the isolated microbead. This bridging existed for all microbead diameters studied. In addition, the side-view images of the ribbon surface indicated that the meniscus partially encapsulates the microbeads, irrespective of diameter. Commonly for microbeads ⩽ 90 μm diameter, reflections of both bead and meniscus appeared on the surface of the gelatin coating, perhaps due to ribbon–microscope horizontal plane misalignment and the reflective optical properties that gelatin exhibits when observed at low angles of viewing. The vertical height of the meniscus increased with increasing spherical diameter. Evaporation of the water content caused recession of the meniscus border over the course of minutes, as seen in the time-lapse images in figures 2(C) and (D). Even as the meniscus contracted toward the ribbon–bead interface, the exposed microbead remained adhered to the ribbon surface. Complete evaporation of the water volume occurred faster for smaller bead diameters (~4 min for 45 μm) than for larger bead diameters (>9 min for 600 μm). The meniscus recession around the larger bead was also accompanied by the lateral movement (~200 μm distance parallel to the XY plane of the ribbon) of the microbead, perhaps due to gravitational effects resulting from a ribbon surface that deviated from the horizontal plane, whereas smaller beads showed no appreciable movement. Thus, the interplay of two forces, surface tension and bead weight, caused the microbead to become adsorbed and positioned on the print ribbon. Microbead diameter only affected the meniscus height and timescale of evaporation, but did not influence meniscus-mediated adsorption of the bead on the ribbon surface.

Transfer of single microbeads

Real-time video feed on the LDW GUI allowed isolated beads to be targeted prior to transfer, and visual verification of a successful transfer, as shown in figure 3(A). The gelatin-based DRL was optically transparent, allowing for identification of beads on ribbon surface. Polystyrene microbeads appeared opaque with a central glare caused by back-illumination. Alginate microbeads appeared transparent, with occasional glares and shadows created by the reflection and refraction of light through the hydrogel material. Isolated beads were aligned with the superimposed crosshairs on the GUI camera screen, where a single laser pulse was delivered upon programmed user trigger. Indications of a microbead ejection in the area of interest included clearance of desired microbead from the video feed, deformation of the DRL, residual laser-induced vapor bubbles and blurred glare of light due to refraction of under-lighting by the dispensed droplet. In our study, microbeads of diameters up to 250 μm were printable. Failed microbead ejections were characterized by the formation of sub-threshold bubbles, the burred repositioning of the targeted bead on the GUI camera screen and the lateral translation of bead on a ribbon surface, as observed in the attempted transfer of a 600 μm diameter polystyrene bead. Corresponding droplets of material on uncoated receiving substrates revealed transferred microbeads with co-ejected gelatin DRL. Gelatin is a thermoreversible hydrogel at 37 °C and has been shown to melt under cell culture conditions [32, 39]. In some transferred spots, the bead appeared off-center in relation to the gelatin droplet location; however, it was not determined whether the ejection dynamics or receiving substrate movement after transfer was the source of this misalignment.

We characterized the rate of successful transfers as a function of bead size to better understand the size capabilities of LDW to print single microbeads. As shown in figure 3(B), the likelihood of transferring microbeads by LDW diminished as the bead size increases. The most common mode of failure for smaller microbeads was the ejection of a gelatin droplet without an accompanying microbead. More notably, the probability of transferring a 600 μm diameter bead was essentially 0%, where laser/materials interaction at the targeted bead area failed to desorb the bead from the ribbon.

Operational parameters that affected the likelihood of single bead ejection by LDW include (1) laser beam cross-sectional area, (2) laser fluence, (3) area of clearance around a targeted bead, (4) environmental conditions at the time of transfer (5) and size of microbead. In order to constrain the complexity of the parameters, we consistently printed microbeads under ambient room conditions (~23 °C, ~40% RH). The cross-sectional laser spot was limited to a roughly circular area (A ~4.90 × 10⁴ μm², φ ~250 μm), as shown in figure 3(C), but could be reduced in size to tailor to each microbead diameter. In comparison, the laser spot covered an area smaller than the field-of-view in the GUI video feed. When a suspension was prepared on the ribbon surface, beads in close proximity to neighboring beads may aggregate due to the surface tension effects caused by combined and receding menisci. Delivery of a single laser pulse to an area of locally high microbead density will cause the transferred spots to contain more than one bead. To minimize entrainment of neighboring beads during the transfer process, isolated beads were targeted such that no neighboring beads appeared in the field-of-view. Thus, we have demonstrated the capability of LDW to transfer single microbeads by restricting the microbead size and defining favorable LDW operational parameters. In the same respect, alginate and mESC-alginate beads were targeted and deposited with favorable conditions identified for polystyrene beads.
**Figure 3.** (A) Top row: Representative in situ camera screenshots of targeted microbeads on the print-side ribbon surface, aligned with the crosshairs that mark the center of a laser beam cross section. Middle row: After the delivery of a single laser pulse, the presence of bubbles and/or light glares in the image indicated transfer of materials from the ribbon to the substrate. In some instances, such as the 600 μm diameter microbead, a bead may fail to eject from the ribbon surface. Bottom row: Corresponding micrographs of ejected material on the substrate confirmed the success or failure of transfer. Ejected droplet may include gelatin, as indicated by the white arrows. Scale bar = 44 μm, unless otherwise noted. (B) The probability of successful single bead transfer as a function of bead diameter. For each discrete diameter studied, a total of 20 attempts were tried. The data were fitted to a binomial curve (R² = 0.954). (C) Subtractive ablation of light-(λ = 193 nm) sensitive ink on a glass slide conferred a 3 × 3 pattern with uniform Cartesian spacing of 750 μm, as defined by the g-code program. Each pattern position contained the typical size of cross-sectional laser beam diameter (φ ~250 μm) used to transfer microbeads. The laser beam has a near-Gaussian energy distribution, an average fluence of 0.44 ± 0.07 J cm⁻² for LDW and a pulse width of 8 ns. Scale bar = 200 μm.

**Patterning of microbeads**

Polystyrene and alginate microbeads were successfully printed into defined patterns by LDW, as shown in figures 4 and 5. To demonstrate additive fabrication of patterns with various-sized microbeads, a programmed definition generated 3 × 3 arrays with uniform Cartesian spacing of 600 μm. Between each laser pulse, isolated microbeads were targeted on the ribbon before the user-triggered delivery of a single laser pulse to eject the desired bead. The additive sequence of printing started at the top right-hand corner, proceeded down the column before starting at the top of the next column and ended at the last position at the bottom left of the pattern, as shown in figure 4. In figure 4(A), direct-writing of 45 μm microbeads resulted in a pattern of discrete transferred spots, near-consistently containing single beads. In figures 4(B) and (C), attempts at transferring 90 and 250 μm diameter beads resulted in partially and randomly populated arrays. The extent of pattern completion varied accordingly with the probability curve for successful single bead transfers established in figure 3(B). Hence, it was easier to achieve complete patterns with 45 μm diameter beads than with 90 or 250 μm diameter beads (probability = 80%, 55% and 35%, respectively). In figure 4(D), suspensions of 45 and 90 μm diameter microbeads were mixed and loaded onto the ribbon before LDW by size selection. Through visual selection, beads were targeted to create a 3 × 3 pattern of alternating small and large beads. Although 45 μm diameter beads transferred more readily than 90 μm diameter beads resulting in a partially populated array, we have shown that LDW is capable of transferring desired polystyrene beads from a heterogeneous source.

LDW was also able to achieve fully populated, ordered patterns of acellular alginate beads, as seen in figure 5(A). Beads desorbed from ribbon with complete fidelity to the defined pattern, with each position consistently containing one microbead. Following transfers, alginate microbeads appeared intact, with smooth peripheral surfaces.
Figure 4. Laser direct-written patterns of polystyrene microbeads on gelatin–alginate matrix-coated substrates. Fully populated 3 × 3 array of (A) 45 μm diameter beads and partially populated 3 × 3 arrays of (B) 90 μm and (C) 250 μm, as defined in figure 3(C). (D) Simple 3 × 3 array of alternating 45 and 90 μm microbeads. The white arrows denote the location of 45 μm diameter beads. The dotted-line circles indicate the location of missing microbeads in a desired pattern. Scale bar = 200 μm.

Printing of mESCs encapsulated in alginate beads

Precise deposition of mESC-alginate beads into defined positions gave rise to spatially discrete, stem cell microenvironments, as shown in figure 5(B). Cell populations remained inside printed microbeads, even after 72 h of culturing. In figures 5(C) and (D), live/dead staining confirmed cell survival and viability in hydrogel microbeads, directly after transfer. The viability of control (directly from suspension with no manipulation) beads was 70 ± 14%. The viability of pipetted beads (randomly patterned and subjected to immobilization steps) was 51 ± 25%. The overall rate of viable cells within laser-direct microbeads was 37 ± 11%, or ∼73% of the value observed in pipetted microbeads. In Student’s t-test assessment, there was no significance difference observed between the viability rates for fabricated and LDW microbeads (p > 0.05). Thus, the laser direct-writing by soft desorption is gentle enough for cell work and the force of impact during microbead ejection does not significantly affect the cell viability. Printed beads were maintained under culturing conditions for 72 h and mESCs proliferated within each discrete microenvironment. LDW microbeads maintained long-term stem cell growth within the hydrogel microenvironments.

Adhesion tests of laser direct-written microbeads

Laser direct-written microbeads may be subjected to post-processing procedures; thus, the preservation of pattern spatial order is critical for addressable positions in the array. Direct-written alginate microbead patterns were immobilized in situ onto alginate–gelatin coated substrates. Gelatin provided the cushion to dissipate the force of bead impact with the receiving substrate surface. In addition, the deformable hydrogel temporarily confined the transferred beads while subsequent beads were additively deposited at room temperature. However, gelatin melted under culturing conditions resulting in loss of pattern stability. The transient effectiveness of gelatin was offset by long-term adhesion of microbead to substrate via divalent (Ca²⁺) cross linking of alginate. Complete polymerization of the thin substrate film occurred on the time-scale of minutes.

In order to assess the adherence of microbeads to the substrate, the position of each bead centroid was measured...
Figure 5. Laser direct-writing hydrogel microbeads into ordered patterns on receiving substrates. Fully populated (A) 3 × 3 array of alginate microbeads, as defined by the pattern shown in figure 3(C). Scale bar = 200 μm. (B) Triangular array of mESCs encapsulated in alginate beads. Pairs of beads are spaced 600 μm apart, with incremental separation spacing of 350 μm in the orthogonal axis. One microbead became dislodged from the pattern during the immobilization process, as shown by the black arrow. Scale bar = 200 μm. (Inset) Pattern definition, as shown by the subtractive ablation of 193 nm wavelength light-sensitive ink on a glass slide. (C) Representative mESC viability assessment in a laser direct-written microbead, as determined by the live/dead staining. (D) Cell viability in microbeads after electric-field driven fabrication, random dispersion (LDW-) on coated substrates and LDW. Values normalized to cell viability after fabrication.

in reference to a fiduciary mark under static and dynamic conditions. As shown in figure 6(A), immobilized microbeads continuously maintained registry to the initial pattern for 24 h under static conditions following LDW and 10 min of ultrasonic disturbances, exhibiting no displacements from the printed pattern. In figure 6(B), the overlapping points corresponding to each identified bead at various time points during ultrasonication indicated the retention of microbeads in their printed positions. As shown in figure 6(C), only 67% of control beads (pipetted onto substrate and immobilized with CaCl₂) maintained their position after 1 min of ultrasonication. Missing later time points conferred dislodgement of microbead from the field-of-view, and shifts in time points corresponding to an identified bead indicated the displacements of the bead over time.
Figure 6. Immobilized mESC-alginate microbeads on substrates coated with alginate–gelatin film. (A) Micrographs (10 ×) of a 3 × 3 pattern that was challenged with 1, 5 and 10 min of exposure to ultrasonic waves. (B) Position of indexed direct-written microbeads from a fiduciary marker at various time points during ultrasonication. (C) Position of indexed randomly deposited beads (control) on a substrate surface at various time points during ultrasonication. Missing data points at later times indicate dislodged beads that drifted out of field-of-view. (D) Field emission SEM images of (top left) a randomly deposited alginate microbead and (bottom left) a single direct-written alginate microbead. The radial deformations of substrate film were artifacts introduced in alginate–gelatin film during processing. Right column: recessed grooves at bead–substrate interface indicating partial embedment of a microbead.
Discussion

The LDW system has the ability to print a user-specified pattern configuration in a CAD/CAM manner, through the deposition of individual microbeads onto a 2D plane with single-bead resolution. Integration of an in situ visualization system enabled viewing of specimens on the same plane as the laser beam focus for the selection and eventual transfer of the desired microbead. The success of single-microbead transfer depended on the formation of a vapor pocket, which is defined by the water volume at the bead–ribbon interface.

The gelatin-mediated adhesion kept the microbead adhered to the ribbon surface, despite complete evaporation of the meniscus. The fact that microbeads did not desorb from the ribbon surface under the force of their own weight meant that a deliberate desorption scheme was required to eject microbeads from the print ribbon onto a receiving substrate. Since the basis of the LDW mechanism hinges on the local evaporation of water that causes vapor formation and bubble expansion that expelled materials from the ribbon [40], the presence of the water meniscus critically affected the success of printing. Microbeads on a dried-out ribbon did not transfer as successfully as beads on a hydrated ribbon. However, excessively wet conditions may cause entrapment of neighboring microbeads during ejection. In addition, the conditions shown in figure 2 did not account for the proximity of a parallel, wet receiving substrate surface within 500 μm of ribbon plane. The local environment between substrate and ribbon may reach ~100% RH and slow the meniscus evaporation rate. In any event, release of microbead from the ribbon required a triggered laser–DRL interaction to facilitate the formation of a vapor pocket that expels the targeted microbead. Future work will focus on the mechanism of laser-induced bubble expansion in the volume of DRL directly above microbeads as a function of bead diameter and laser fluence.

We surmise that larger microbeads may displace larger volumes of DRL material during the embedment process of the ribbon preparation, reducing the effective volume of a laser-sensitive material to absorb the incident laser energy. As a result, it may lead to a lower vapor pressure to eject the bead. In addition, larger masses of larger microbeads may require higher laser energy to overcome the surface tension. We were able to achieve transfers of single microbeads, irrespective of microbead material identity, because the ejection momentum was provided by the laser–DRL interaction.

Alginate microbeads were chosen as the model material because the hydrogel is a soft material (Young’s modulus, $E = 66$ kPa), and may potentially incur physical damage at the point of impact with the receiving substrate. Occasionally, a deposited microbead exhibited a burst shape, indicating surface disruption and escape of uncross-linked content. However, the surface damage was minimized by coating the substrate with an alginate–gelatin mixture that acts as a cushion to dampen the force of bead impact. We note that alginate beads smaller than 200 μm in diameter transferred more successfully than polystyrene beads of comparable diameters, perhaps due to differences in material–meniscus interaction for small beads that may enhance the ejection mechanism. This physical phenomenon will be investigated in a future study.

The use of an alginate–gelatin film as an immobilization scheme has several advantages. First, the thin film is optically transparent, making it useful for transmission light imaging. Second, rapid, reversible cross links form in the presence of Ca$^{2+}$ and rapid depolymerization occurs in the absence of Ca$^{2+}$ [41]. Thus, the long-term pattern registry may be preserved with the introduction of calcium cations. Conversely, microbeads may be released from pattern positions through the removal of calcium. Finally, the chemical treatment does not destroy cells or microbead structural integrity. Although these advantages may be applied to virtually any microbead on the receiving substrate surface, they fail to explain why direct-written beads maintained registry more successfully than randomly dispersed control beads.

It is possible that the impact of ejected beads during the transfer process enhanced the adhesion of microbeads onto the substrate film. Partial embedment of the alginate bead into the substrate film resulted in a pronounced physical deformation of the alginate–gelatin film at the bead–substrate interface, as shown in figure 6(D). Through SEM-aided observations, the cavity at interface appeared larger for direct-written beads than beads randomly pipetted onto the surface. Generally, the impact force during droplet landing increases the embedment of microbeads, augmenting the contact area between the peripheral bead surface and the hydrogel film on the receiving substrate [42]. The larger surface area may correspond to an increased number of cross links between the alginate at the bead surface and the alginate in the film upon the addition of Ca$^{2+}$ ions. The increased interaction is a possible explanation as to why the ejected beads maintained registry, whereas pipetted control beads did not. Elucidating the impact-induced mechanical force on the microbead and substrate interaction requires further study for better patterning performance. Overall, the alginate-based immobilization scheme effectively retained laser-direct-written alginate microbeads on the receiving substrate in their printed positions.

Future applications of spatially-ordered mESC-alginate beads include use as proximal co-cultures for paracrine signaling studies, microbioreactors for cell-based lab-on-a-chip technology and patterned cell-laden 3D microenvironment subunits for engineered tissue constructs. Since alginate is chemomechanically tunable, one can create a library of biomimetic microenvironments for the systematic study of mESC differentiation or study elastic moduli-directed mESC differentiation. A unique application for cell-laden alginate microbeads as a cell-culturing platform relies on the characteristic chemically reversible Ca$^{2+}$-mediated cross links. One can construct a dynamic cell-culturing platform with the ability for on-demand release of mESCs from beads through use of citrate, a Ca$^{2+}$ chelator. Thus, cells growing in a 3D hydrogel microenvironment can easily be transferred to the surrounding 2D or 3D scaffold, while maintaining pattern registry. Patterns of drug-loaded hydrogel microbeads can be generated for the controlled release from spatially customizable gradients. The capability to direct-write alginate microbeads provides exciting new venues and functionality for hydrogel microbeads.
Conclusions

LDW was able to target and deposit single microbeads to achieve real-time spatial ordering. Gentle transfer maintained the physical bead integrity and stem cell viability in patterns of discrete microenvironments. Long-term spatial order was preserved, even when challenged with ultrasonic disturbances. LDW is capable of systematically direct-writing cells encapsulated in hydrogel microenvironments into customizable patterns for potential long-term studies. Future work will focus on the characterization of the transfer mechanism and the development of patterned microbioreactor systems for biological studies.

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