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Controlled packing and single-droplet resolution of 3D-printed functional synthetic tissues

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3D-printing networks of droplets connected by interface bilayers are a powerful platform to build synthetic tissues in which functionality relies on precisely ordered structures. However, the structural precision and consistency in assembling these structures is currently limited, which restricts intricate designs and the complexity of functions performed by synthetic tissues. Here, we report that the equilibrium contact angle (θ_DIB) between a pair of droplets is a key parameter that dictates the tessellation and precise positioning of hundreds of picolitre-sized droplets within 3D-printed, multi-layer networks. When θ_DIB approximates the geometrically-derived critical angle (θ_c) of 35.3°, the resulting networks of droplets arrange in regular hexagonal close-packed (hcp) lattices with the least fraction of defects. With this improved control over droplet packing, we can 3D-print functional synthetic tissues with single-droplet-wide conductive pathways. Our new insights into 3D droplet packing permit the fabrication of complex synthetic tissues, where precisely positioned compartments perform coordinated tasks.

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The packing of space-filling polyhedra in three dimensions (3D) is a common theme in nature, from the atomic packing of molecules in crystal structures, to the regular arrays of cells in tissues, to the macroscopic tessellation of honeycombs created by bees. In these examples, distinctive properties and precise functions arise from controlled arrangement and patterning of their subunits. Similarly, the controlled close packing of deformable spheres is critical for fabricating synthetic tissues that are designed to mimic the complex and cooperative structures and functions of living tissues. In this area, networks of picolitre-sized droplets separated by droplet interface bilayers (DIBs) hold significant promise because of discrete compartmentalisation, inherent connectivity and communication between subunits.

A DIB forms when two aqueous droplets in oil, each stabilised by a monolayer of lipids, are brought together and form a bilayer at the droplet–droplet interface (see Supplementary Note 1). Networks of droplets can be made based on this interfacial interaction. Patterned networks of droplets have been produced by mechanical placement, microfluidics, optical tweezers and magnetism. The most powerful approach for fabricating extended patterned networks has been droplet-based 3D-printing, which provides 3D design capabilities, automation and scalability.

When two aqueous droplets come into contact and form a DIB, they deform from their initial spherical shape to form a flat bilayer interface between them. At equilibrium, the area of the lipid bilayer is reflected by a contact angle (θDIB) measured at the point where the spherical surfaces of the two droplets meet at the flat lipid bilayer interface. When aqueous droplets pack in 3D, each makes multiple droplet–droplet contacts (by forming DIBs with its neighbours), resulting in the deformation of the spherical droplets into polyhedra. Therefore, when building a 3D network, it is crucial to understand the parameters that direct packing and droplet deformation, and thereby affect printing resolution and fidelity. At present, for example, the occurrence of printing defects dictates that conductive signalling pathways in large networks (>100 droplets) must be designed to be more than 2–3 droplets wide to ensure continuity. However, single-droplet-wide signalling pathways would be feasible if synthetic tissues could be patterned at single-droplet resolution.

Various studies have aimed to understand the close packing and subsequent deformation of adhesive droplets in highly concentrated oil-in-water emulsions. Recently, microfluidic systems have been developed to investigate how droplets pack in extended 2D sheets and how small clusters of adhesive droplets self-organise under flow. Most relevant to our printed system—where droplets are placed in specific arrangements—are studies by Princen et al. on how the value of the contact angle between pairs of adhesive oil-in-water droplets controls droplet packing in small 2D and 3D assemblies. Based on Princen’s observation and early work on adhesive water-in-oil emulsions, we hypothesised that the value of θDIB between pairs of droplets would be a major geometrical constraint on the packing of hundreds of deformable aqueous droplets interfaced by lipid bilayers (see Supplementary Note 3 for discussion on geometrical constraints in droplet networks). For DIBs, θDIB reflects the balance of the surface tensions between the lipid monolayers and the lipid bilayer (see Supplementary Note 1 for discussion on the thermodynamics of DIB formation). Therefore, factors that alter the surface tensions (such as the compositions of the aqueous, lipid and oil phases), change θDIB. Furthermore, we hypothesised that at an optimal θDIB, droplets will regularly arrange in a hexagonal close-packed lattice (one of the optimal, ordered arrangements for close packing of spheres) and space in a network would be completely filled by tessellated droplets assuming regular polyhedral geometry. Here, we demonstrate experimentally how, by controlling θDIB, we can create regular hexagonal close-packed (hcp) lattices from hundreds of 3D-printed picolitre-sized droplets. Furthermore, we apply regular and controlled droplet packing to fabricate synthetic tissues with single-droplet-wide features in 3D, using an automated and readily scalable technology.

**Results**

θDIB depends on lipid and oil compositions. To determine how θDIB depends on lipid and oil compositions, we measured the contact angle (Fig. 1a) of pairs of 75 nL droplets containing phosphate-buffered saline (PBS, pH 7.2) under various conditions. At a total lipid concentration of 1 mM, θDIB depended upon both the volume fraction of silicone oil in a mixture of undecane and silicone oil (φSIL) and the lipid composition (φPOPC). We found that θDIB is directly proportional to both φSIL (at 1 mM DPhPC) (Fig. 1b–d, f) and φPOPC (at φSIL = 0.65) (Fig. 1g), and that the maximum value of θDIB of 90° was approached at φSIL = 0.65 and φPOPC = 1.00 (Fig. 1e). Temperature (4–60 °C), droplet volume (0.52–200 nL), and total lipid concentration (1–4 mM) had no significant effect on θDIB (Supplementary Fig. 1; see Supplementary Note 1 for further discussion).

We used θDIB values from droplet pairs formed over a range of lipid and oil compositions to obtain a planar regression that accurately describes the 2D linear relationship of θDIB with respect to φPOPC and φSIL (Fig. 1h):

$$\theta_{DIB} = \frac{0.930\phi_{SIL} + 0.368\phi_{POPC} - 0.238}{0.009}$$

Eq. (1) allowed us to predict θDIB from the φSIL and φPOPC values used for 3D-printing droplet networks.

3D-printed networks contain regular and irregular packing. To explore the relationship between θDIB and the packing arrangements of droplets, we constructed 3D droplet networks comprising hundreds of picolitre-sized aqueous droplets (PBS, 100 μm diameter, =524 pl volume) using our laboratory-built 3D printer. To form a network, 224 droplets were automatically generated at a droplet ejection frequency of 0.5 s–1, and positioned line-by-line and layer-by-layer according to an hcp design (x, y and z dimensions of 7, 8 and 4 droplets, respectively) (Fig. 2a; Supplementary Note 2). The droplet networks were formed on plasma-treated quartz, unless otherwise stated.

To quantify packing arrangements, we imaged the interconnected phospholipid bilayers and monolayers by confocal microscopy (Fig. 2b; Supplementary Fig. 2) and assigned packing types based on Delaunay triangulation (Fig. 2c; Supplementary Fig. 3, see “Methods”). Horizontal cross-sections of the first (bottom), second, third and fourth (top) layers in droplet networks showed the 2D droplet packing within the plane of each layer (Fig. 2b). Layers two, three and four were difficult to resolve because of insufficient laser penetration and aberrations caused by the water–oil interfaces in the underlying layers (Fig. 2b; see Supplementary Note 4 for further discussions). Therefore, we quantitatively assigned packing types (see below) to areas of the first layer’s 2D packing structure. We later discuss how the packing arrangements of the first layer dictate the packing of droplets in the upper layers.

By analysing 129 printed networks (Supplementary Fig. 3), we found two prevalent regular packing arrangements that we classified as ‘hexagonal’ and ‘square’ (Fig. 2c). These correspond to ‘hexagonal close-packed’ (hcp) and ‘body-centre cubic’ (bcc)
Fig. 1 | The contact angle depends on the lipid and oil compositions. a Schematic of a pair of aqueous droplets forming a droplet interface bilayer (DIB) in a lipid-in-oil solution, and the definition of the equilibrium contact angle ($\theta$$_{DIB}$). b–e Bright-field and fluorescence microscopy overlays of droplet pairs formed with 1 mM DPhPC and $\varphi$$_{SIL}$ values of 0.20 ($\theta$$_{DIB} = 6.0 \pm 0.7^\circ$) (b), 0.50 ($\theta$$_{DIB} = 26.5 \pm 1.7^\circ$) (c), 0.80 ($\theta$$_{DIB} = 53.4 \pm 0.8^\circ$) (d) and with 1 mM POPC and $\varphi$$_{SIL}$ = 0.65 ($\theta$$_{DIB} = 85.3 \pm 3.8^\circ$) (e). Scale bars = 150 µm. In each image, the right droplet contains 10 µM Atto488 to demonstrate that a bilayer has formed and compartmentalises the bilayer-impermeant dye. f A plot showing the linear dependence of $\theta$$_{DIB}$ with respect to $\varphi$$_{SIL}$ for 1 mM DPhPC (linear regression $R^2 = 0.99$) (see Supplementary Table 1). g A plot showing the linear dependence of $\theta$$_{DIB}$ with respect to $x$$_{POPC}$ at $\varphi$$_{SIL}$ = 0.65 (linear regression $R^2 = 0.99$) (see Supplementary Table 2). h A plot of the 2D linear dependence of $\theta$$_{DIB}$ with respect to both $\varphi$$_{SIL}$ and $x$$_{POPC}$ (regression plane $R^2 = 0.99$) (see Supplementary Table 8). The total lipid concentration was 1 mM. Data points that lie above and below the regression plane are in magenta and cyan, respectively. For all experiments, the aqueous phase was PBS at pH 7.2. Each data point in f–h is the mean of $n$ = 3 contact angle measurements, and the error bars represent the standard deviation. When error bars are not visible, they have standard deviations smaller than the data symbols. For individual $n$ values, see Supplementary Table 24.

3D lattices, respectively. We also found irregular packing arrangements, which we classified as ‘amorphous’ or ‘not packed’ depending on whether the neighbouring droplets were in contact or not (Fig. 2c).

In addition to packing arrangements, we quantified three types of defects in the first layer: fraction of the total area occupied by oil inclusions (i.e., pockets of oil trapped among surrounding droplets, Supplementary Fig. 3b, i), number of droplets that had fallen from upper layers to the bottom layer during printing (Supplementary Figs. 2a–c and 3f), and variation in droplet size (Supplementary Fig. 3k) (see “Methods” for a detailed explanation of the packing analysis).

$\theta$$_{DIB}$ controls droplet packing in 3D-printed networks. We first determined how the packing of 3D-printed droplet networks depends on $\theta$$_{DIB}$ (calculated from Eq. (1)). When varying $\varphi$$_{SIL}$ at constant lipid composition (1 mM DPhPC), we printed droplet networks at $\theta$$_{DIB}$ values that ranged from 9.9° ($\varphi$$_{SIL}$ = 0.35) to 57.5° ($\varphi$$_{SIL}$ = 0.8) (Fig. 2d–h).

At low $\varphi$$_{SIL}$ (0.35, i.e., low $\theta$$_{DIB}$), the predominant arrangement type in droplet networks was no-packing (0.53 ± 0.08 area fraction) (Fig. 2d, i, n). Networks printed under this condition also showed abundant droplet rolling from the upper layers (65 ± 18% droplet excess) (see Supplementary Fig. 3i) and a large fraction of oil inclusions (0.18 ± 0.01 area fraction) (Supplementary Fig. 3i). In contrast, at high $\varphi$$_{SIL}$ (0.80, i.e., high $\theta$$_{DIB}$), we observed abundant amorphous packing (0.40 ± 0.05 area fraction) (Fig. 2h, m, r) and droplet excess (61 ± 24%) (Supplementary Fig. 3j), together with a small fraction of oil inclusions (0.03 ± 0.01 area fraction) (Supplementary Fig. 3l).

Interestingly, at $\varphi$$_{SIL}$ = 0.59 ($\theta$$_{DIB}$ = 35.3°), we found the largest fraction of hexagonal packing (0.43 ± 0.06 area fraction, Fig. 2f, k, p), together with low extents of oil inclusions (0.06 ± 0.01 area fraction) (Supplementary Fig. 3j), droplet rolling (13 ± 6% droplet excess) (Supplementary Fig. 3j), and droplet size variation (8.2 ± 1.5 coefficient of variation) (Supplementary Fig. 3k). This $\theta$$_{DIB}$ of 35.3°, equal to approximately half the dihedral angle of a regular tetrahedron (70.5°, 3 s.f.), corresponds to the geometrically optimal condition for packing of droplet networks.
Fig. 2 $\theta_{\text{DIB}}$ affects the packing of droplets in 3D-printed networks. a Images of a 3D-printed droplet network (7 × 8 × 4 droplets in x, y and z directions) as each layer (1 to 4) is formed. b Horizontal cross-sections of layer 1 (bottom), layer 2, layer 3 and layer 4 of a droplet network imaged by confocal microscopy. Lipid bilayers and monolayers are visualised with Atto550M. c Examples of packing types in the first layer of 3D-printed networks. Our classification method draws triangles (indicated by black and highlighted white triangles) between the centres of three neighbouring droplets and assigns the local packing type of each triplet based on the triangle geometry (Delaunay triangulation). Droplet triplets are classified as packed in a hexagonal (yellow), square (red) or amorphous (cyan) fashion, or not packed (blue) (see "Methods"). d–m Confocal microscopy images (d–h) and overlays of the packing analysis based on Delaunay triangulation (i–m) of the first layer of 3D-printed droplet networks at increasing $\phi_{\text{SIL}}$ values (corresponding to increasing $\theta_{\text{DIB}}$). Yellow, red, cyan and blue triangles represent the packing types defined as hexagonal, square, amorphous and no packing, respectively. Scale bar = 100 μm. n–r Quantification of hexagonal (yellow), square (red), amorphous (cyan) and no-packing (blue) area fractions in the first layer of 3D-printed droplet networks at increasing values of $\phi_{\text{SIL}}$. In n–r, each bar is the mean of $n > 3$ networks (see Supplementary Table 21 for individual n values and Supplementary Fig. 3); error bars represent the standard deviation.
calculated critical contact angle ($\theta_c = 35.3^\circ$, 3 s.f.) required to exclude the continuous oil phase enclosed by four droplets, when their centres are positioned at the vertices of a regular tetrahedron (Supplementary Note 3 for derivations and experimental evidence). When $\theta_{\text{SIL}}$ was increased only slightly to 0.60 ($\theta_{\text{SIL}} = 36.3^\circ$), we observed a significant drop in the hexagonal packing area fraction to 0.31 ± 0.05 (Supplementary Fig. 3e, see Supplementary Fig. 5a for accuracy of measurements).

We find that hcp is maximised in 3D-printed droplet networks when $\theta_{\text{DIB}} = \theta_c$ because, at this contact angle, the tetrahedral arrangements of droplets are maintained in the position imposed by the printing nozzle with minimal distortions. In a pair of droplets forming a DIB, the adhesive energy of the system ($\Delta F$; defined as the work required to form a lipid bilayer per unit area) increases with increasing bilayer area, and therefore with increasing $\theta_{\text{DIB}}$ (Supplementary Eq. 1f, Supplementary Eq. 3). Consequently, larger values of $\theta_{\text{DIB}}$ in 3D-printed networks lead to increasingly cohesive and compact droplet assemblies. However, when a DIB is formed, the two droplets reduce their centre-to-centre distance as a consequence of the deformation of the droplet interface had reached a non-equilibrium value of 30°. Since this value of $30^\circ$ is optimal for 2D hexagonal packing of droplets (Supplementary Note 3), the matching of the printing frequency and the fast phase of DIB formation allowed the first layer to pack hexagonally before the second layer was printed (Fig. 3b, the time taken to print a layer was $t_{\text{layer}} = 225$ s). The slow phase in DIB formation allowed the slow development of hcp from the hexagonal packing in the first layer, as the non-equilibrium contact angle reached its final equilibrium value of $36.3^\circ$ ($= \theta_c$). We further confirmed this by observing that deviations from a printing frequency of $0.50 \text{s}^{-1}$ significantly reduced hexagonal packing in the networks printed at the same lipid–oil composition (Fig. 3d).

Packing arrangements localise in specific regions. Our optimised conditions for obtaining hcp lattices increased network regularity. To study whether ordered and disordered patches localised to specific regions of printed networks (e.g., centre versus edges), we overlaid 2D cross-sectional images of the first layers of droplet networks printed at $\theta_{\text{SIL}} = 0.55$ and $x_{\text{POPC}} = 0.13$ (Fig. 4a), and mapped the occurrence of different types of packing onto an idealised map of the first layer (see “Methods”) (Fig. 4b).

We observed that droplets were mostly hexagonally packed throughout the networks, while square, amorphous and no-packing arrangements were confined to the edges (Fig. 4b). Due to their positions, droplets at the edges of the networks were coordinated to less than six neighbouring droplets in the first layer (Fig. 4c), and as a result often exhibited irregular packing (amorphous and no packing) (Fig. 4b). Conversely, droplets that were coordinated to six neighbours predominantly exhibited hexagonal packing (Fig. 4b). Once a small cluster of droplets is hexagonally packed during the printing process, it may serve as a nucleation event, propagating hexagonal packing to neighbouring areas and thereby producing extended hexagonal regions (Fig. 4c).

In summary, droplets surrounded by six others predominantly formed extended hexagonally packed regions. In contrast, irregular packing was confined to the periphery of the printed networks, where droplets were surrounded by fewer than six neighbours.

Packing arrangements propagate upwards. By using overlays of 2D cross-sections of the first, second and third layers, we observed the spatial propagation of packing arrangements from the first layer to the upper layers (Fig. 4d–h). For hexagonal lattices (Fig. 4d), droplets in layers 1 and 3 were in alignment with each other, but offset with respect to droplets in layer 2 by centre-to-centre distances equal to the circumradius of a unit hexagon (defined in Fig. 4d), in accordance with an hcp lattice (Fig. 4e).

Within hexagonal regions, we sometimes found droplets in the...
third layer offset with respect to both layers 1 and 2, corresponding to face-centred cubic arrangements (Fig. 4d).

Non-hcp regions were also propagated from the first layer to the upper layers. For instance, sections of square packing in the first layer propagated into body-centred cubic packing arrangements (Fig. 4f, g). In disordered sections of the first layer, and generally when $\theta_{\text{DIB}} > \theta_s$ (amorphous packing), we found that disordered packing arrangements propagated into the upper layers (Fig. 4h).

In summary, packing arrangements in the first layer propagated into the upper layers, stressing the importance of regular packing of droplets in the first layer. The surface onto which the network was printed played an important role in supporting an ordered first layer. Droplets printed on plasma-treated quartz formed an adhesive patch (likely a lipid bilayer$^{31}$) with the surface, which constrained droplets in position while printing. In contrast, droplets printed on non-treated quartz or rough glass formed less regular arrangements (Supplementary Fig. 7).

**Fig. 3** The kinetics of contact angle equilibration affects hexagonal packing. For all conditions in a–e, $\theta_{\text{DIB}} = 36.3^{\circ}$ (calculated from Eq. (1)). The $\varphi_{\text{SIL}}$ and $x_{\text{POPC}}$ values in b–d are $\varphi_{\text{SIL}} = 0.60$ and $x_{\text{POPC}} = 0.00$ (cyan), $\varphi_{\text{SIL}} = 0.55$ and $x_{\text{POPC}} = 0.13$ (purple), and $\varphi_{\text{SIL}} = 0.52$ and $x_{\text{POPC}} = 0.20$ (magenta). a Optical microscopy images of two droplets (75 nL) forming a DIB after contact. The non-equilibrium contact angle ($\theta$) increases with time (0–900 s) until the equilibrium contact angle ($\theta_{\text{DIB}}$) is reached ($t = 1800$ s). Scale bar = 150 µm. The images correspond to timepoints along the purple profile in b. b Plots of $\theta$ versus time for droplet pairs. Plots are the mean of $n=3$ repeats for each condition, and error bars represent the standard deviation. The grey dashed lines correspond to timepoints relevant to printing 3D droplet networks: $t_{\text{fast}}$ (0.50 s), $t_{\text{drop}}$ (2.00 s) and $t_{\text{slow}}$ (4.00 s) indicate the time intervals between consecutive printed droplets at fast ($t_{\text{fast}} = 2.00$ s), standard ($t_{\text{prop}} = 0.50$ s) and slow ($t_{\text{drop}} = 0.25$ s) printing frequencies; $t_{\text{drop}}$ (225 s) is the time it takes to print a single layer at a printing frequency of 0.50 s$^{-1}$. c A bar chart of the hexagonal packing area fraction of 3D droplet networks printed at a droplet ejection frequency of 0.50 s$^{-1}$. The hexagonal area fraction under the purple condition was significantly greater than for the other two conditions (one-way ANOVA with Tukey’s multiple comparison test) (see Supplementary Table 15). d Plots of hexagonal packing area fractions in the first layers of 3D droplet networks generated at different printing frequencies ($f_0$). The frequencies marked in grey are the inverse of the timepoints marked in b (i.e., $f_0 = \tau_0^{-1}$, where $\tau_0$ is the time interval between the ejection of two consecutive droplets). For statistical tests, see Supplementary Table 16. e A confocal microscopy image and overlay of Delaunay triangulation of the first layer in a 3D-printed droplet network ($7 \times 8 \times 4$; x, y, z) at $\varphi_{\text{SIL}} = 0.55$ and $x_{\text{POPC}} = 0.13$ (purple condition in b–d) at a printing frequency of 0.50 s$^{-1}$. Scale bar = 100 µm. Each data point in the graphs c and d is the mean of $n > 3$ repeats, and error bars represent the standard deviation. *, ** and *** indicate p-value <0.05, 0.01 and 0.001, respectively. For individual $n$ values, see Supplementary Tables 22 and 23.
3D replicas of tessellated droplet shapes confirm hcp. To confirm the hcp of droplets in 3D-printed networks, we imaged the space-filling shapes adopted by the droplets within a network. The final shape of a droplet is determined by $\theta_{\text{DIB}}$ and by the number of surrounding droplets and their locations. Optical aberrations caused by water–oil interfaces prevented us from directly imaging 3D shapes of individual fluorescent droplets within printed networks by optical microscopy (Supplementary Note 4 and Supplementary Fig. 8). Instead, we created fluorescent-poly(ethylene glycol) hydrogel replicas of droplets in networks, dispersed these replicas in PBS and reconstructed their 3D geometries from $z$-stacks acquired by confocal microscopy (Fig. 5a–c; see “Methods”). Droplet replicas were often attached to each other, thereby representing the 3D packing structure we observed in droplet networks (Fig. 5d; Supplementary Fig. 9 and Supplementary Movies 1 and 2). In hexagonally packed regions of droplet network replicas, a droplet in the second or third layer was surrounded by 12
droplets, which formed 12 DIBs—producing the space-filling trapezo-rhombic dodecahedron ($D_{3h}$) (Fig. 5d) expected for hcp. Horizontal cross-sections of these polyhedra showed a regular hexagon at the midpoint (Fig. 5h), with two aligned equilateral triangles near the top and bottom (Fig. 5g, i). In contrast to what we saw for hcp, horizontal sections of a polyhedron in a face-centred cubic lattice—a rhombic dodecahedron ($O_h$)—would show two equilateral triangles, near the top and the bottom of the polyhedron, rotated by 60° with respect to each other. Droplets at the bottom of a network (touching the
Single-droplet-wide features in synthetic tissues. Finally, we applied our findings on regular droplet packing to the fabrication of synthetic tissues with high-resolution functional features in 3D. In biological systems, complex functionalities emerge not only from the cell types present but also from the coordinated interaction of simpler functions performed by specialised cells organised in defined architectures. When designing synthetic tissues, we take inspiration from biological systems to develop artificial systems with behaviour not restricted to biological or biomimetic functions.

To demonstrate how spatially controlled droplet packing defines functionality, we designed a synthetic tissue whose functionality directly emerged from the correct positioning of individual droplets (by applying $\theta_{\text{DIB}} = \theta_c$ and confining high-resolution patterns to the centre of the construct) (Fig. 6). Specifically, this construct featured two separate, electrically conductive, single-droplet-wide pathways (composed of droplets containing the ion-permeable membrane protein α-hemolysin (αHL), which were patterned in 3D within a network of non-conductive droplets (which did not contain αHL). Based on the pore formation mechanism of αHL in lipid bilayers, the two conductive pathways in our design were separated by three insulating droplet layers to electrically isolate the two pathways from each other. The two pathways spanned the construct within different droplet layers: one horizontally at the bottom of the network (within layer 1, connecting A to C in Fig. 6b, c) and one vertically at the top of the network (within layer 5, connecting B to D in Fig. 6b, c). Imperfections in the droplet lattice would lead to either interruption of the electrical signal along the single-droplet-wide conductive pathways, or crosstalk between the two pathways designed as distinct (see circuit model in Fig. 6c). Therefore, electrical recordings would give us a direct readout of the precisely controlled packing of droplets in this construct.

By confocal microscopy, we observed the designed configuration of the single-droplet-wide conductive pathway at the bottom of the construct (Fig. 6d). We could not resolve the upper conductive pathway because of the imaging limitations discussed above (Supplementary Note 4). Interestingly, we observed that αHL-containing droplets showed a smaller $\theta_{\text{DIB}}$ compared with droplets not containing αHL. We attributed this decrease in $\theta_{\text{DIB}}$ to the interaction of αHL with the lipid monolayers and bilayers, leading to changes in surface tension. However, the neighbouring buffer droplets, for which $\theta_{\text{DIB}} \approx \theta_c$ packed on an hcp lattice (in the centre of the construct) and constrained the αHL-containing droplets to an hcp lattice, which consequently maintained precise interconnection of the single-droplet-wide conductive pathway (Fig. 6d).

To confirm the correct functionality of the synthetic tissue, we performed electrical recordings. When a potential of ±50 mV was applied, we detected ionic currents flowing through the bottom and top single-droplet-wide pathways, i.e., from A to C ($i_{\text{AC}}$) and from B to D ($i_{\text{BD}}$) (Fig. 6e). By contrast, we detected no ionic current when we applied a potential between A and D or B and C ($i_{\text{AD}}$ and $i_{\text{BC}}$), demonstrating that the two orthogonal pathways were spatially and functionally distinct, in accordance with our design. Taken together, these experiments demonstrate that functional synthetic tissues can be created at single-droplet resolution (100 μm diameter, ≈524 PL voxel volume) in three dimensions using our dropletprinter. To our knowledge, this is the first example of an automated system generating such well-controlled droplet packing and patterning on the micrometre scale.

Discussion
In summary, we found that 3D-printed droplet networks adopted hcp lattices when $\theta_{\text{DIB}}$ in droplet pairs approximated the geometrically derived critical angle ($\theta_c$) of 35.3° (Fig. 2). Deviations from this critical angle led to networks that were either loosely packed ( $\theta_{\text{DIB}} < 35.3°$ ) or tightly packed and distorted ( $\theta_{\text{DIB}} > 35.3°$ ), both with increased lattice defects. We also noted a greater extent of hexagonal packing when the printing frequency was matched to the rapid phase of DIB formation to allow regular 2D hexagonal packing to form in the first layer before the upper layers were printed. The rapid phase of contact was followed by the slow formation of 3D hcp droplet lattices as the non-equilibrium contact angle approached $\theta_{\text{DIB}} \approx \theta_c$ (Fig. 3). In addition, regular hexagonal packing of the first layer was crucial because packing arrangements propagated into the upper layers (Fig. 4). In hcp regions, droplets formed space-filling trapezohedral dodecahedra that featured 12 DIBs with the surrounding droplets (Fig. 5). Our findings are applicable to any other assemblies of compartmentalised systems—such as adhesive giant unilamellar vesicles or protein compartments—in which structural order is required to build synthetic tissues with precise functionalities.

Based on our improved control over the packing of droplets within 3D-printed networks, we can fabricate 3D-printed droplet networks with intricate designs at high resolution (e.g., tubular structures, Supplementary Fig. 11). We exemplified this by building a synthetic tissue containing two single-droplet-wide conductive pathways with a minimal separation of three insulating droplet layers, confirming that we are able to pattern functional synthetic tissues with single-droplet-resolution features in 3D (Fig. 6). This level of precision was not achieved at the periphery of the 3D-printed constructs, where most printing defects and irregular arrangements of droplets were confined (Figs. 4, 6). However, our observations also suggest that regular packing at the periphery of 3D-printed droplet networks might be further improved by 'annealing' steps after the printing process (i.e., cyclical decrease and increase in contact angles), or by templating the droplet packing lattice using patterned surfaces.

Our findings show that it is possible to reproducibly pattern droplet networks with single-droplet precision by using an automated and readily scalable technology. Our work provides new knowledge about the parameters that drive droplet packing and opens up new possibilities, such as the fabrication of increasingly complex synthetic tissues that can reproduce the structurally coordinated interactions of living cells. This improved understanding will also allow the fabrication of precisely and reproducibly constructed 3D-printed structures containing eukaryotic and prokaryotic cells to study tissue development, cellular ecology and disease models.

Methods
Lipid–oil solutions. Lipids were purchased from Avanti Polar Lipids and stored as stock solutions in chloroform at a concentration of 25 mg mL$^{-1}$ at −20 °C in 1.5 mL Teflon-capped glass vials (Supelco). Lipid stock solutions were used within a month of preparation. Undecane and silicone oil AR20 (Sigma-Aldrich) were filtered before use through 0.22 μm filters (Corning) under vacuum. Lipid–oil solutions were prepared by evaporating desired amounts of lipid in chloroform (1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)) under a slow
Fig. 6 Synthetic tissues with features patterned at single-droplet resolution. a Maps of the first (bottom), second, third, fourth and fifth (top) layers of a 3D-printed synthetic tissue in which two conductive, single-droplet-wide pathways containing αHL (in yellow) are patterned in 3D within a network of non-conductive droplets (in grey). b A computer model displaying the 3D architecture of the synthetic tissue. The two single-droplet-wide pathways span the synthetic tissue, one horizontally at the bottom (connecting A to C), and one vertically at the top (connecting B to D) of the network. c Simplified diagram of the synthetic tissue, and equivalent circuit model. d Bright-field and fluorescent microscopy overlay of the synthetic tissue. Droplets in the single-droplet-wide conductive pathways contained α-hemolysin (60 µg mL⁻¹), 25 mM Tris-HCl (pH 7.6), 1 M NaCl and 10 µM Atto488 fluorophore (false coloured in yellow). Scale bar = 100 µm. e Electrical recordings of the ionic currents flowing through the two single-droplet-wide conductive pathways connecting A to C (\(i_{AC}\)) and B to D (\(i_{BD}\)) upon application of the voltage protocol shown in f. When a potential of ± 50 mV was applied, changes in ionic currents of +25.6 ± 1.4 pA (at positive potential) and −25.6 ± 1.5 pA (at negative potential) were observed for \(i_{AC}\), and of +19.4 ± 1.2 pA (at positive potential) and −19.8 ± 1.3 pA (at negative potential) for \(i_{BD}\). Conversely, we recorded non-significant changes in ionic current for the same applied potentials between A and D (\(i_{AD}\)) or B and C (\(i_{BC}\)). \(i_{AD}\): +1.8 ± 1.3 pA and −1.8 ± 1.4 pA (at positive and negative potentials respectively); \(i_{BC}\): +0.9 ± 1.1 pA and −0.9 ± 1.1 pA (at positive and negative potentials, respectively).
stream of nitrogen while rotating by hand in isopropanol-cleaned glass vials to produce an even lipid film. The film was dried under vacuum for 30 min followed by addition of oils at a desired ratio and sonication for 30 min before use. Lipid–oil solutions were used within a week.

**Aqueous solutions.** Phosphate buffer saline (PBS, pH 7.2) (Sigma–Aldrich) was filtered through a 0.22 µm filter (Millex) before use. Where indicated, Atto488 and Atto550M (Sigma–Aldrich) were dissolved in PBS at 10 µM and 3 µM, respectively. Fluorescent dyes were diluted in PBS from stock solutions in DMSO (Sigma–Aldrich). Stock solutions were prepared at concentrations of 1–6 nM.

**Forming droplet interface bilayers.** Droplet interface bilayers (DIBs) were formed in computer numerical control (CNC)-machined (Roland monoFab SRM-20) 3 × 3 well arrays made from poly(methyl methacrylate) (PMMA) (Supplementary Fig. 10). Typically, 200 µL of lipid–oil solution was added to the well array followed by injections (using a 0.5 µL Hamilton syringe) of two 75 µL PBS droplets into each well. The droplets were left separate in the lipid–oil solution for 15 min to complete monolayer formation at the oil–water interface. Following this, droplets were brought together using gravity by tilting the well array. Once a DIB had formed, it was left for an hour to reach the equilibrium contact angle (θ_Eq).

The same protocol was applied to form droplet assemblies comprising two-dimensional (2D) triplets and three-dimensional (3D) tetrahedrons, with the exception of manually positioning droplets by using a thin plastic wire. Temperature experiments were performed using a Tokai Hit<sup>TM</sup> Leica TPX (Type HF) Thermo Plate. Contact angle measurements were taken after one hour at a set temperature. Droplet pairs were kept hydrated during the course of heating by using wet paper towels to maintain a saturated humidity and prevent evaporation of the aqueous droplets. Droplets of 100 µm in diameter (0.52 nL in volume) were generated using the 3D printer.

**Imaging the change in contact angle over time.** A DIB was formed between two droplets (∼75 nL) in CNC-machined PMMA wells that consisted of two layers (at different heights) connected by a slope with an angle of 17° (Supplementary Fig. 10b). One droplet was positioned at a small angle of the track, while the other was rotated to the top. By using a pipette tip, the top droplet was pushed to roll down the track so that it came into contact with the bottom droplet. The increase in contact angle over time, once the two droplets made contact, was imaged by optical microscopy (HCX PL FLUOTAR 10×/0.30a, brightfield at 60 µm frames<sup>−1</sup>). Experiments were run for a maximum of 20 min after two droplets had made contact.

**Preparing quartz cuvettes.** Custom quartz cuvettes were purchased from Starna Scientific at dimensions of 20 × 10 × 10 mm (x, y, z). Cuvettes were used for surface studies and as a container for 3D-printing droplet networks. Cuvettes were cleaned by sonication in 5% (v/v) Decan 90 in Milli-Q water and ethanol for 15 min (with plenty of rinsing with Milli-Q water in between and as the last step). Cuvettes were treated in an O<sub>3</sub> plasma cleaner for 4 min before printing and surface studies. Roughened cuvettes were achieved by incubating the cuvettes in 8 M NaOH for 7 days.

**3D-printing droplet networks.** Our custom droplet 3D printer is extensively detailed in previous work<sup>4</sup>. In brief, a piezoelectric transducer transmits controlled pressure impulses to a chamber filled with Milli-Q water. The chamber is connected to a glass nozzle (with a tip diameter of approximately 150 µm) from which the aqueous printing solution is ejected. To prevent mixing of the printing solution with the water inside the chamber and nozzle, an undecane oil plug of ∼5 µL was used to provide a seal. The nozzle was then immersed into PBS solution contained in the quartz cuvette, which was positioned on a digitally synchronised using a custom-made software developed in LabVIEW. Printing was followed by injection (using a 0.5 µL Hamilton syringe) of two 75 nL PBS droplets into each well. The droplets were left separate in the lipid–oil solution for 15 min to complete monolayer formation at the oil–water interface. Following this, droplets were brought together using gravity by tilting the well array. Once a DIB had formed, it was left for an hour to reach the equilibrium contact angle (θ_Eq).

Contact angles were calculated from microscopy images using a custom-written script on MATLAB<sup>®</sup> (Mathworks, Natick, MA) to measure relevant radii and centre-to-centre distances.

**Classifying printed network regions.** To quantify statistics of the droplet networks, a 2D plane from the confocal stack of each 3D network was manually selected to maximise the fluorescent signal from the lipid bilayers between droplets of the bottom layer of each print. This image was then segmented into separate droplets and oil inclusions, initially by a ridge detection algorithm<sup>41</sup> and then by manual verification and cleaning. The resulting segmented networks were analysed using MATLAB<sup>®</sup> (Mathworks, Natick, MA). In the first stage, we classified contiguous regions of the network as either droplets or oil inclusions. This was done on a morphological basis: droplet areas were required to be between 0.4 and 10 times the area of the median region, and to have a face area to convex hull area ratio of at least 0.9. This second condition ensured that only regions that were largely convex were counted as droplets. Other regions were classified as oil inclusions. Droplet overlap in the first layer (the number of droplets that fell from the upper layers to the first layer) of the networks was calculated from (N – N<sub>o</sub>) / N<sub>o</sub>, where N<sub>o</sub> is the number of droplets in the first layer and N<sub>t</sub> is the number of expected droplets in the first layer (Supplementary Fig. 2a–c). N<sub>o</sub> = 72, when a perfectly packed network of 7 × 8 × 4 (x, y, z) droplets is formed; the first layer commonly appeared with a dimension of 8 × 9 × 4 (x, y, z) droplets because of droplets falling from the second and third layer (Supplementary Fig. 2a–c). The variation in droplet size (coefficient of variation) was calculated from the standard deviation of droplet area within a network divided by the average droplet area.

Bias in network classification was avoided by performing manual network adjustments blind to the experimental conditions used in each print.

**Classifying packing types.** To estimate the proportion of packing types within each network, we first generated a mesh over the print by performing a Delaunay triangulation using the centres of all regions classified as droplets as the input points. A convenient property of this triangulation method is that the circumsphere of each triangle contains no other points; each triangle therefore represents a triplet of neighbouring droplets and preserves localised geometrical information about their arrangements.

The plot of the bivariate distribution of the largest angle of each triangle (θ<sub>max</sub>) vs triangle area normalised by the average droplet area (A) for all prints revealed two distinct clusters of triangles, one corresponding to hexagonally packed droplet regions (θ<sub>max</sub> = 60°) and one corresponding to square-packed droplet regions (θ<sub>max</sub> = 90°) (Supplementary Fig. 3d). Reasoning that these represented stable configurations of droplets for certain printing
parameters, we classified each triangle using these clusters. Four packing categories were used: ‘hexagonal’ (closely packed triplets of droplets arranged as equal-sized triangles), ‘square’ (closely packed triplets of droplets arranged as right-angled triangles), ‘amorphous’ (closely packed triplets of droplets of intermediate arrangement) and ‘no packing’ (triplets of droplets that are not close-packed). For a hexagonal classification, $60^\circ \leq \theta_{\text{max}} < 67^\circ$. For an amorphous classification, $67^\circ \leq \theta_{\text{max}} < 89^\circ$. For a square classification, $83^\circ \leq \theta_{\text{max}} < 97^\circ$. In addition, for all of these classes, 0.25 $\leq$ C $\leq$ 0.75. Triangles that fell outside of these ranges were classified as no packing (Supplementary Fig. 3d).

Some elongated ‘sliver’ triangles were classified as being a member of one of the closely packed classes, despite being composed of widely spaced droplets. Closer analysis of these triangles revealed that they were generally composed of two closely packed droplets and a single more distant droplet, together forming a skinny near-isosceles triangle. We therefore applied an additional constraint based on the perimeter of each triangle normalised by the average droplet radius ($P$). Since for perfect hexagonal packing $P = 3\sqrt{3}$, and for a perfect square packing $P = 4 + 2/\sqrt{2}$, we set the constraint $P_{\text{closely packed}} = 0.0545 \theta_{\text{max}}$ for the closely packed classes based on a linear interpolation between these two points. Our chosen $y$-intercept for this constraint (3.73) is slightly greater than the theoretical $y$-intercept from the interpolation, allowing triangles with small deviations from the perfect packing geometry to be classified as closely packed while still ensuring classification of sliver triangles as no packing.

**Localisation of packing types in printed networks.** For the dataset with maximal hexagonal packing ($\phi_{\text{frac}} = 0.55$ and $\chi_{\text{opf}} = 0.13$), 2D network images were manually registered to a reference network image using the Interactive Rigid Transform plugin for ImageJ. This plugin only permits rotational and translational transformations, so the geometry of the network is preserved during registration. Regions of registered images were then classified as droplets or inclusions, and droplet triple triangle patterns were extracted and classified as described above. For each print $i$ and each of the four packing types $j$, binary images $B_i(R_j)$ were generated indicating the presence or absence of each packing type at each spatial location $R$. A rough heatmap for each class was then calculated as the sum of the binary images over all prints. $H(R) = \sum B_i(R_j)$.

To create the regularised heatmaps (Fig. 4b), an idealised version of the print geometry was manually registered to the reference network image. The boundaries of the droplets in this registered idealised image were used to define a set of hexagonal bins $h$, associated with a collection of pixel locations $r_i$. For each bin, the sum of all pixels within its boundaries for each of the four rough heatmaps $h_{i,j} = \sum H_i(R_j)$ was then calculated. Finally, the normalised packing proportion $s_j$ for each bin and each packing type was calculated as $s_j = h_{i,j}/\sum h_{i,j}$.

**Electrophysiological recordings.** For the electrophysiological recordings shown in Fig. 6, a lipid-oil solution composed of 2 mM DPhPC in an undecane-silicone oil mixture with $\phi_{\text{frac}} = 0.43$ was used. The aqueous solution used for both conductive droplets probe the phase diagram of jammed granular matter. Proc. Natl Acad. Sci. USA 108, 4286–4291 (2011).

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Author contributions
A.A., R.K.K. and H.B. conceived and designed experiments, and wrote the paper. A.A., R.G.A. and R.K.K. performed the experiments. O.J.M. wrote custom software for the image analysis of droplet networks and performed the image analysis. C.M. performed and aided in microscopy experiments. V.R.S. performed and aided in the electrical recordings. I.C. optimised conditions for α-hemolysin purification and use. M.T.C. performed α-hemolysin expression and purification.

Competing interests
The authors declare no competing interests.

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