Creating paper scaffolds.

Step 1: Choice of paper

A wide variety of paper can be used, depending on requirements concerning the microscopic structure and desired thickness of the paper. The use of general purpose paper (e.g., printer paper) is not recommended as it generally contains fillers and other additives which make it less suitable. In the paper we use Whatman Chromatography 1 paper and we have successfully experimented with various kinds of filter paper and lens paper. Supplementary Table 1 in Derdra et al. *PLOS One* 2011 provides a list of suitable paper types and their material properties.

Step 2: Cutting paper

Paper can be cut to shape in many ways. Laser cutting provides a convenient, fast, and precise means of cutting paper with < millimeter accuracy. Common laser cutters take graphics created in e.g., Adobe Illustrator, Inkscape, or CorelDraw as input (file requirements depend on the make and model of the laser cutter). We have used a Versa Laser VL-300, an Epilog mini, and a Trotec Speedy 400, all instruments produced the desired result with comparable accuracy (for the designs presented in the paper). Please note that paper can catch fire during laser cutting, as this should be avoided cutting should be aborted when smoke and/or flames are visible.

After cutting, the paper scaffolds can be autoclaved in a standard autoclave and subsequently dried in a glass container (with lid) in an oven set to 80°C. Autoclaved and dried scaffolds should be stored in dry environment.

Step 3: Preparing the scaffold

Depending on the experimental design, the paper scaffold can either be suspended (in air) on a wire frame to restrict dispersal to the paper matrix as in the dendritic network assay described in the paper
(step 3A), or submerged in liquid medium allowing dispersal through the liquid as well as through the paper as in the archipelago assay described in the paper (step 3B).

**Step 3A: Scaffolds suspended on a grille**

Preparation of the grille: Bacteria may disperse through the thin liquid film that forms between wet paper and a supporting surface. To prevent this, we suspend the liquid saturated paper on a wire frame (grille) in experiments where dispersal should be restricted to the paper matrix. A grille minimizes contact between the wet paper and supporting surface and thus restricts dispersal to the liquid saturated paper.

We construct the grille using a metal frame (x,y dimensions should be larger than the paper substrate) and PEEK (Polyether ether ketone) tubing. We use PEEK tubing for the wires because of its biocompatibility and smooth surface. Any water resistant tape can be used to secure the tape to the frame at approximately 5 millimeter intervals (depending on the shape of the paper scaffold, and the paper type used, a larger pitch can be used).

**Step 3B: Scaffolds secured to a surface and submerged**

When the experimental design requires paper scaffolds to be in liquid allowing dispersal between different paper scaffolds through liquid, it is important to secure the paper scaffolds to a surface to prevent movement of the scaffolds during incubation. Securing the scaffolds on a cover slide facilitates microscopy and can be done using molten Parafilm. To this end, cut Parafilm in the same shape as the paper scaffolds in use, remove the wax paper backing, place the Parafilm shapes on a cover slide, and place the corresponding paper scaffolds on top. Melt the Parafilm by placing the assembly on a hotplate set to 80°C for approximately 40 seconds and remove from the hotplate to cool to room temperature. If necessary, excess Parafilm surrounding the paper scaffolds can be cut using a razor blade. Once the scaffold has cooled to room temperature, nutrients can be seeded in the paper islands by pipetting on solutions containing the desired amounts and drying the scaffolds.
Inoculation and incubation

Step 4: Saturating the paper

Before inoculating the scaffolds with a culture, the scaffolds have to be saturated with liquid medium. If the scaffolds will be suspended on a grille, scaffolds should be submerged in liquid medium for 5 minutes. After submerging, excess medium should be allowed to drip from the paper scaffold, the scaffold can be positioned on the grille, and the grille + scaffold can be placed in a chamber with saturated humidity (i.e. a petri dish with water soaked tissue). Prior to inoculation, the scaffold on the grille should acclimatize in an incubator set to 37°C for 30 minutes.

In case a grille is not used, the cover slide holding the scaffolds can be submerged in a beaker containing medium (when colonization from a bulk liquid is intended), or a liquid cell can be created on the cover slide (as in the archipelago assay). A liquid cell can be created by placing an adhesive barrier around the scaffolds, filling the enclosed area with medium, and closing the cell using a cover slip. We obtained good results using rectangular Gene Frame adhesives (17 x 28 x 0.25 mm, Thermo Fisher Scientific) as the enclosing barrier yet many other strategies (such as quick cast PDMS, or other fast setting silicones) work as well.

Step 5: Inoculation

Depending on the experimental design, paper scaffolds can be inoculated with a wide variety of bacterial consortia. Care should be taken that no unwanted displacement of liquid is induced when applying the bacterial culture to the paper surface. To avoid this, we do not inoculate using a pipette, but rather dip a 1 μL inoculation loop in the bacterial pre-culture and place the seeded inoculation loop in contact with the inoculation zone of the paper scaffold. This method efficiently inoculates the paper scaffold yet does not induce flow in the liquid saturated paper.

Step 6: Incubation

Inoculated paper scaffolds can be incubated in a standard incubator. Scaffolds suspended on a grille should be enclosed in a chamber with saturated humidity at all times to prevent drying. Scaffolds enclosed in a liquid cell can be briefly taken out of the incubator for imaging and returned to the incubator for
longer incubation. We do not recommend this for suspended scaffolds as drying during imaging will compromise the experiment.

Imaging

Step 7: Microscopy

Paper has rather high autofluorescence (especially in the blue) which complicates imaging of fluorescent bacteria, yet can be taken advantage of to image the exact structure of the paper. To minimize the contribution of out of plane (auto)fluorescence we used confocal microscopy to image the scaffolds. Best results are generally obtained when the imaging substrate is in liquid, as a consequence scaffolds in a liquid cell can be imaged without modification whereas scaffolds suspended on a grille should be fixed prior to imaging. After fixation, scaffolds can be imaged in a liquid cell as used for the archipelago assay.

Step 7A: Fixation

Suspended scaffolds can be fixed by removing the scaffold from the grille and submerging it in 4% formaldehyde in phosphate buffered saline (PBS) for 15 minutes. After fixation scaffolds should be washed 3 times with PBS. Scaffolds can be imaged in PBS + 50% glycerol in a liquid cell (cover slide, Gene Frame, cover slip). Fixation has the added benefit that scaffolds do not have to be imaged immediately yet can be stored prior to imaging allowing replica scaffolds to be inoculated, incubated, and fixed simultaneously while imaging can be done sequentially at a later stage.

Assessing community composition with qPCR

After incubation the community composition can be assessed by extracting genomic DNA from the paper scaffolds and quantifying the abundance of community members using quantitative PCR. If desired metagenomic or barcoded sequencing may also be used to assess community composition.

Step 8: DNA extraction

DNA can be extracted from appropriately cut pieces of paper through standard protocols. We have obtained good results using the NucleoSpin Tissue kit (Macherey-Nagel) following the manufacturer’s
protocol. Many available DNA extraction kits have a suitable protocol, e.g. to extract DNA from filter paper or FTA cards, that will be appropriate.

**Step 9: qPCR**

For the dendritic assay described in the paper we performed qPCR on an Eco Real-Time PCR system (Illumina) using SYBR Green PCR Master Mix (ThermoFisher Scientific). The GFP/RFP *E. coli* community used in the paper can be assessed using primer sets GFP: 5' - GCCA ACTCTGAAAAAGTCATGCT - 3' (forward), 5' - CATGGCCAACACTTGTCACT - 3' (reverse), RFP: 5' - CCTGAA GGGCGAGATCAA - 3' (forward), 5' - TGGCCATGTA GGTGGTCTTTG - 3' (reverse). Prior to performing qPCR on gDNA retrieved from paper scaffolds, the qPCR assay should be benchmarked using defined test samples, e.g. communities mixed at known fractions.

To facilitate the processing and visualization of qPCR data from commonly used qPCR machines (AB StepOne, and Biorad CFX) we have created python scripts that are available at:

https://github.com/felixhol/qPyCR