Supplemental File 1 – Experimental Design Considerations

There are many aspects to consider when designing a CMO-DPAC experiment.

Feature dimensions: What size features are required? Transparency-style photomasks tend to have a minimum feature size of about 10 µm diameter, and the maximum feature size can be millimeters or centimeters long. To pattern single cells, use DNA spots 10 – 20 µm in diameter (ideal spot size will depend on size of cell). The number of cells adhering to a given DNA spot will increase as the size of the spot increases. The shape of the features can be adjusted to meet the needs of the experiment. Since the DNA is patterned by a photomask, any shape that can be drawn in a computer aided drawing program can become a pattern for cells.

Spacing of features: How close should the cells be to each other? To study the interaction between a pair of cells, pattern the single cell sized spots spaced only by a 10 µm gap. To study the interaction between multi-layered aggregates of cells, a spacing of 100-300 µm may be more appropriate. On the other hand, if each individual feature should be treated as an individual replicate and interactions between cells is undesirable, features should be spaced out several hundreds of microns apart. It can be challenging to align multiple oligos with high precision, so consider providing some extra space between features for different cell types. Overlapping features are not recommended, as it results in the presence of both oligos at the overlap region (Supplemental Figure 3), and therefore could have either cell type adhere to that region.

Arrangement of cells relative to each other:
To create multilayered structures (Figure 5C), start by patterning the first cell population (bottom layer) onto the slide, then add cells that were labeled with an Adapter Strand complementary to the Adapter Strand of the first cell population. After washing away excess cells, the first population of cells will have the second population attached to the sides and top. To add a third layer, add cells labeled with the same Adapter Strand as the first cell population, and repeat for any additional layers, alternating between the two complementary Adapter Strands. The size of the multilayered aggregate will increase with each layer, both upwards and outwards. Since the aggregate is only attached to the slide at the bottom layer, aggregates of 3 or more layers can get top-heavy and prone to being washed away (Supplemental Figure 2C). Thus, we'd recommend using a base DNA spot of at least 30 µm for large multilayered cell aggregates.

If the different cell populations will be arranged in the same layer (Figure 6), orthogonal oligos will be required. For example, for patterning three different cell types, use Adapter Strands A, B, and C (Supplemental File 2). Additionally, multiple orthogonal oligos (complementary to those Adapter Strands) will need to be photopatterned sequentially. Fiducial markers on the aldehyde slide will be required to align the slide with the photomask so that the different features are spaced correctly relative to each other. Fiducial markers can be created in a variety of ways, including by deposition of thin metal films[1], using acetone-resistant ink or glass scratches to draw fiducial markers, aligning the edges of the photomask with the edges of the

slide (use a mask aligner for best results), or by preserving a photopatterned feature from the first round of photopatterning by using tape to protect it from the acetone wash. When patterning the cells onto the slide (Step 6), start with the least abundant cell population by number of features and work up to the most abundant cell population.

Culture conditions of patterned cells: After patterning the cells, the cells can then be cultured in 2D or 3D. The choice of how to culture the patterned cells will depend on the cell behaviors of interest and the timescale of the experiment. Culturing in 2D is simpler and faster – once the cells are patterned, put the slide in a Petri dish and add media. However, the DNA does not keep the patterned cells in place for more than a few hours. Eventually, membrane turnover and the deposition of ECM onto the glass surface will allow cells to migrate and break the original pattern. 3D cell culture opens up a lot of opportunities to observe 3D cell migration, tissue morphogenesis, and the interactions between cells and their ECM. Cells can be cultured for at least a week within the hydrogel, though they too will deviate from the initial pattern as they move and grow. One advantage of this method is that the patterned cells within the hydrogel are all in a single imaging plane, making it easy to observe cell behaviors over time. The downside is that the embedding of cells in the hydrogel for 3D cell culture increases the experiment time (by about 1.5 hours) and increases the failure rate of the experiment, as the transferring of the cell-laden hydrogel from the slide to the second hydrogel droplet can be challenging and requires practice.

Experimental Complexity: The more unique cell types that are patterned, the longer a given experiment will take, both with the photopatterning of the slides and with the patterning of the cells. As many as ten orthogonal oligos have been photopatterned successfully onto the same slide[1]. However, this can take several days of photopatterning to complete. Moreover, since it takes about 10 minutes per cell population to pattern the cells onto the slide and wash excess cells out (Step 6), increasing the number of cell populations in an experiment will increase the overall length of the experiment and potentially decrease cell viability. We have patterned up to 6 cell populations (2 photopatterned oligos x 3 layers each) at once, and a maximum of 4 cell layers at a time. More complexity is certainly possible, but there are certainly tradeoffs in terms of experimental effort and the likelihood of success. Complicated CMO-DPAC experiments can be more complicated to achieve and will likely require some optimization from the user.

We'd recommend learning CMO-DPAC by starting with just one or two cell populations, and then include additional cell populations if desired after the technique has been mastered.

1. Scheideler, O.J. *et al.* Recapitulating complex biological signaling environments using a multiplexed, DNA-patterning approach. *Science Advances*. **6** (12), eaay5696, doi: 10.1126/sciadv.aay5696 (2020).