## **Supplementary Information**

## 1. Concentration profile of the gradient

Since the concentration profile is invariant in direction of the flow channels (perpendicular to the diffusion chamber), a one-dimensional description of the gradient can be adopted. If the supply channels maintain a constant source and sink concentrations  $c_{source}$  and  $c_{sink}$ , and the length of the diffusion chamber is L, then the diffusion dynamics is given by the following diffusion-reaction-equation

$$\frac{\partial c}{\partial t} = D\nabla^2 c - kc$$

where D is the diffusion coefficient and k is the decay constant. As initial condition we assume the concentration to be zero ( $c(x,0)=0 \ x \in [0,L]$ ), and as boundary condition we choose a constant source concentration ( $c(0,t)=c_{source}$ ) and constant sink concentration ( $c(L,t)=c_{sink}$ ). The decay of a molecule is assumed to be first order and is characterized by its decay constant *k*.

If there is no decay, a linear gradient is formed in steady state.

$$c(x) = c_{\text{source}} + \frac{c_{\text{sink}} - c_{\text{source}}}{L}x$$

Here, the slope of the gradient (its steepness) is given by  $(c_{sink}-c_{source})/L$ . If there is decay the profile can be described by:

$$c(x) = c_{\text{source}} \cdot e^{-\sqrt{\frac{k}{D}}x} + \frac{c_{\text{sink}}}{L}x - \frac{c_{\text{source}}}{L} \cdot e^{-\sqrt{\frac{k}{D}}L}x$$

This implies that the concentration of sink and source, the length of the diffusion chamber, the diffusion coefficient and the decay constant characterize the shape of the gradient. With the geometry given in the produced device the only controllable element is the concentrations of source and sink channels. One can have further control over the concentration profile by modifying the length of the diffusion chamber.



**Supplementary Figure 1** A small pressure imbalance in the source and sink channels can result in cross-chamber flow. Here, the pressure of the sink channel filled with PBS was set to 0.1 bar and the pressure of the source channel was set to 0.2 bar. A FEM simulation (left) predicts that the diffusion chamber will be flushed by the food dye, which will leave the device through the sink outlet. The concentration of the diffusive agent plotted here (Red: high concentration, green: low concentration). The simulation was then experimentally validated using red food dye (right). The chamber is fully flushed by food dye and a interface between the foodye and PBS is formed in the buffer region. Even smaller pressure imbalances can lead to such crossflow, which are easily induced during experiments when both source and sink inlets are open.



**Supplementary Figure 2** (A) Photobleaching of FITC-Dextran. A cell culture chamber was filled with 300  $\mu$ M FITC-Dextran (M=40 kg/mol) and continuously exposed to light. By assuming a first order decay of the intensity, the specific decay rate was determined as  $k_{phot}$ =0.000662 s<sup>-1.</sup> (B) FEM simulation of the gradient at steady state including the first-order kinetics of the photo bleaching. (C) Linear relation between FITC-Dextran (M=40 kg/mol) and the measured intensity. The results were obtained by filling a cell culture chamber with a defined concentration and measuring the fluorescence intensity. (D) Validation of the Beer-Law for red food dye (Allura Red, FD&C red 40). The results were obtained like in (C) but instead of using a fluorescent source the bright-field picture was used, as reported in [M.H.V. Werts. *et. al., Lab Chip*,2012,**12**:808-820].



**Supplementary Figure 3** (A) Simulation of flow lines at the cell-chamberchannel boundary. The heat plot shows the velocity magnitude for a channel that is permanently flushed and at steady state with respect to flow. The arrows indicate the direction of flow. The blue lines represent the flow lines through the channel-chamber boundary of 400 traced samples. (B) Averaged magnitude of flow within the buffer zone at different positions. Constant flow at steady state is assumed. The plot shows that the diffusion chamber area that begins at position 300 um is diffusion dominated. (C) Measured flow lines using 2 um polystyrene beads at a constant flow of 5 mm/s are shown.





**Supplementary Figure 4** Quantification of the FITC-Dextran gradient using a 2 minute maintenance cycle. Pictures were taken for 17 hours every 30 minutes. Since the concentration is constant parallel to the supply channels the profile could be reduced to a 1D curve. The steady state is reached after 80 minutes. Different lines show measurements form experimental hour 2 to 17.



**Supplementary Figure 5** (A) Shows a sketch of the automation protocol and reuse of the chip device. (B) Cell attachment measurement of 3T3 fibroblast.



**Supplementary Figure 6** This time-dependent FEM simulation shows the maximum concentration error (% variation from the steady state gradient profile) at any given location in the diffusion chamber after both sink and source valves are closed. A linear gradient across the chamber was used as initial condition. The gradient starts decaying due to diffusion of molecules from high to low concentration regions, and the concentration profile flattens in time. This is equivalent to the experimental procedure where the inlet valve for the sink and source channels were kept closed after establishing the gradient. The simulation was repeated for different diffusion constants, from  $20 \ \mu m^2$ /s (blue curve at the bottom) to  $400 \ \mu m^2$ /s (pink curve at the top), with a step size of  $20 \ \mu m^2$ /s. The experimental conditions we used (2 minute maintenance cycle, D=  $100 \ \mu m^2$ /s) results in around 7% maximum error in this simulation, where as it was experimentally measured to be 5% (Fig 1G).



**Supplementary Figure 7** Same plot as figure 4E but without the spatial component and separated as in Figure 3.