Supplementary figures and movie legends

Supplementary Figure 1 – Preparation of agarose containers. (**a**) The container template is composed of an aluminum tube and an aluminum plunger. The plunger tip can be conical, rounded, or flat.. (**b**) Liquid high-melting agarose is sucked into the template by pulling the plunger. After agarose gelation, the agarose container is removed from the template and stored in PBS. (**c-d**) The cell-gel mixture (yellow, red) is injected into the agarose container by employing a glass capillary or a syringe with a small gauge needle.

Supplementary Figure 2 – Mounting the agarose container. Prior to an experiment, the container is inserted into the custom-made plastic holder and fixed by a pin. A magnetic connector for the attachment to the xyz-stage of the TC-SPIM is inserted into the holder from the top. A protective Teflon foil is mounted between the plastic holder and the magnetic connector. The foil seals the open upper part of the TC-LSFM chamber and prevents contamination of the culture medium during the time-lapse imaging, without interfering with any movements of the specimen holder.

Supplementary Figure 3 – Zooming photographs of an agarose container containing a cell-collagen mix. (**a)** Scale bar 1 mm. (**b)** Zoom in of the container in (a) containing the collagen-cell mix. Each spot is a MDCK aggregate. The images were recorded with a stereomicroscope. Scale bar 500 μm. (**c)** MDCK cysts inside the same container shown in (a) and (b). Image recorded with a Zeiss Axiovert 40 phase-contrast microscope. Scale bar 100 μm.

Supplementary Figure 4 – Preparation and mounting of an agarose container in the TC-LSFM chamber. (**a**) The plunger is pushed out of the tube and the polymerized agarose container is visible. **(b)** The container is separated from the plunger by immersing it in PBS and by gently pushing with bent sharp-tip forceps. **(c)** The container can be easily manipulated with the forceps. **(d)** The vertical agarose container is held by the petri dish wall before filling it with the cell-gel mix. **(e)** The assembled LSFM chamber. The detection objective lens is visible in the window on the right hand side. The agarose container containing the 3D cell-gel mix can be seen in the center. **(f)** A closer view of the agarose container through the side window.

Supplementary Figure 5 – Experimental temperature distribution within the TC-LSFM chamber and temperature stability over time. (**a**) The temperature was measured inside the entire chamber volume of 15 ml by moving a temperature sensor sequentially through a

three-dimensional grid (a 5-point cross measured in 23 planes spaced 1 mm apart). A continuous medium flow of 300 µl/min was maintained to reproduce the actual conditions during a time-lapse recording. The picture shows the temperature distribution in the central plane of the chamber. The central "orange" volume varies by less than 0.5°C. (**b**) The temperature was measured with the internal PT-100 sensor placed in a cavity within the chamber wall. The cavity is positioned at the specimen's height (**Figure 1**, **Supplementary Figure 4**). In total 18,000 temperature values were measured, one per second over a period of five hours.

Supplementary Figure 6 – Measurement of the pH in the TC-LSFM-chamber over time and color change of the medium in the gas exchanger box. (**a**) The pH in the chamber was measured by a glass electrode (Orion 9107BNMD, pH logger Orion 3 Star) by recording 140 points over 20 minutes (one measurement every 10 sec, precision 0.001, relative accuracy ±0.002). The time line is divided into three parts, which cover the first 2.5 min, another 7.5 min and the final 10 min. The pH values shown in the initial period (period **I** of the plot) were measured with the silicon tube spiral placed within the gas exchanger box filled with a 5% CO_2/air mixture. The period **II** of the plot shows the fast rise in the pH once the silicon tube spiral is removed from the box and exposed to laboratory air. The period **III** shows the pH decrease once the silicon tube is placed back into the box containing the 5% CO2/air mixture. (**b**) The silicon tube spiral was placed back into the gas exchanger box containing the CO_2/air mixture at t = 10 minutes. The pH of the cell culture media measured in the TC-LSFM-chamber was 7.7, as indicated by the purple color of the cell culture media flowing in the tube. The media contains phenol red as pH indicator. At t = 16 minutes the pH in the TC-LSFM-chamber was 7.4 and the indicator's color turned yellowish.

Supplementary Figure 7 – Three-dimensional rendering of the aggregate in Figure 4. The volume segmentation allows assessing the morphological changes of the MDCK cell aggregate over time. The parameters that can be evaluated are the total cyst volume, the volume of the internal lumen (red), as well as the overall aggregate shape. One can observe that the cyst is not perfectly spherical, but flattened along the z-axis. The three-dimensional data processing and rendering was performed with the software AMIRA. The xy (left) and xz (right) views are shown at each time point. Scale bar 50 µm.

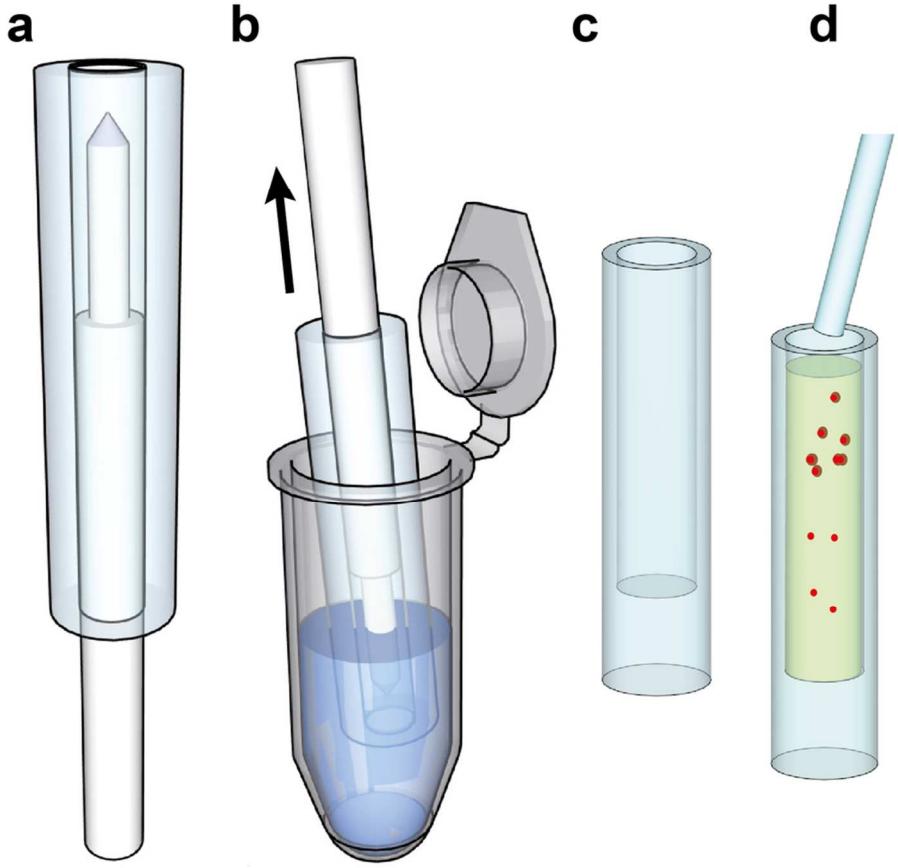
Supplementary Figure 8 – Comparison between one MDCK cyst imaged outside and inside the agarose container. (a) MDCK cysts were cultured in collagen within an agarose container for a period of three-days, in a cell culture incubator at 37°C and 5% CO_2. Thereafter, the collagen slab with the embedded cysts was extracted from the container and imaged with an epifluorescence microscope Zeiss Axiovert 135. Left: phase contrast image. Right: fluorescence image. Marker: F-Actin-GFP. (b) MDCK cysts were cultured in collagen within an agarose container for a period of three-days, in a cell culture incubator at 37°C and 5% CO_2. Thereafter, the agarose container containing the collagen-embedded cyst was mounted in the TC-LSFM and several cysts were imaged. Left: phase contrast image. Right: fluorescence image. Marker: F-Actin-GFP. In both (a) and (b) the same objective lens Carl Zeiss water dipping N-Achroplan 40x 0.8 NA was employed.

Supplementary Movie 1 – 4D microscopy of the development of an individual MDCK cyst. The same cyst as in Figure 4 is shown in the video. At each time point as in Figure 4 a three-dimensional stack of the developing cyst consisting of 50 slices spaced 0.5 μm is shown. Transmitted-light images of the same cysts are also shown, in order to visualize the surrounding environment of the cyst. Objective lens: Carl Zeiss water dipping N-Achroplan 40x 0.8 NA.

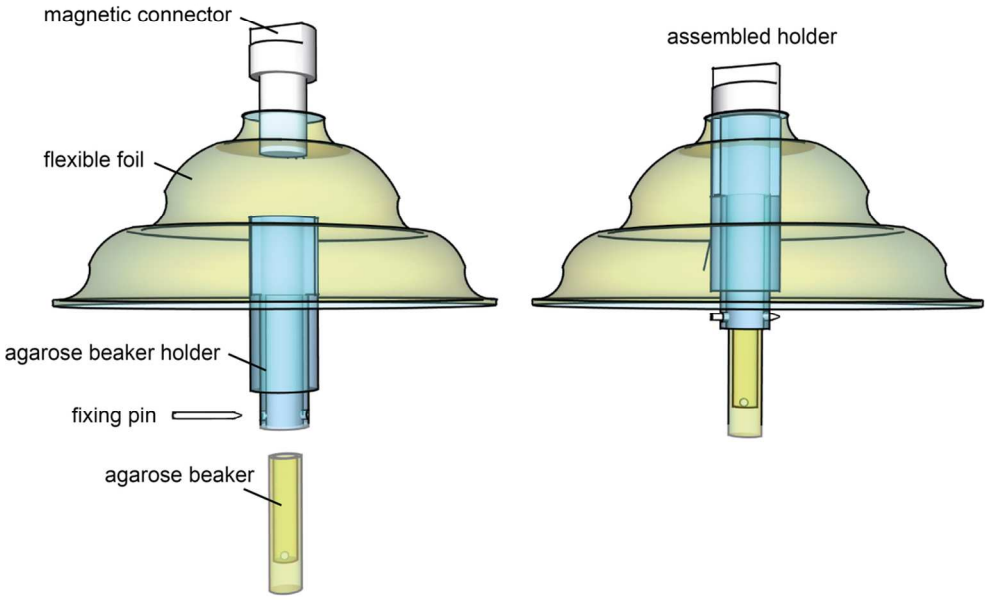
Supplementary Movie 2 – 4D microscopy of a single MDCK cell (expressing YFP-H2B) developing into a multicellular aggregate shown in Figure 6a. Images show average projections of a 3D stack in the xy-, yz-, and xz-dimensions. Frames 1 to 43 show the YFP-H2B fluorescent signal (2 frames per second, where each frame was recorded every 2 hours) and frames 44 to 66 show the bright field signal (2 frames per second, where each frame was recorded every 4 hours). Scale bar 10 μm.

Supplementary Movie 3 – Imaris representation of tracked nuclear centroids (white cubes with cell IDs) shown in Figure 6b. The elapsed time in the bottom left represents the time points (2 hours). The color bar on the bottom right represents the time of the tracks. Scale bar 10 μm.

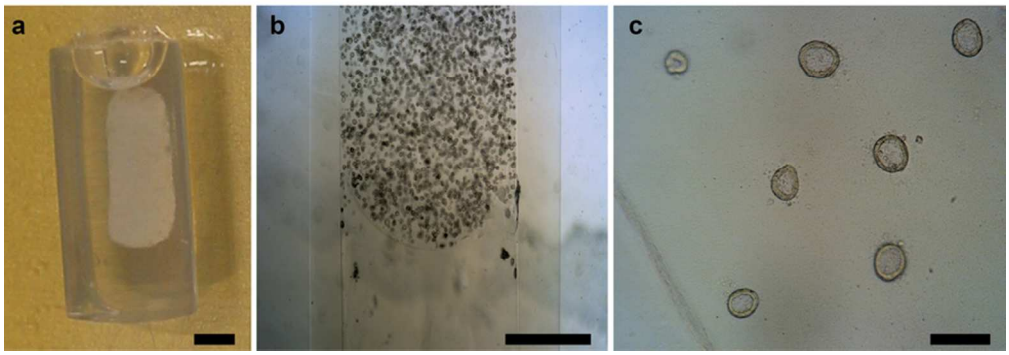
Supplementary Figure SF1



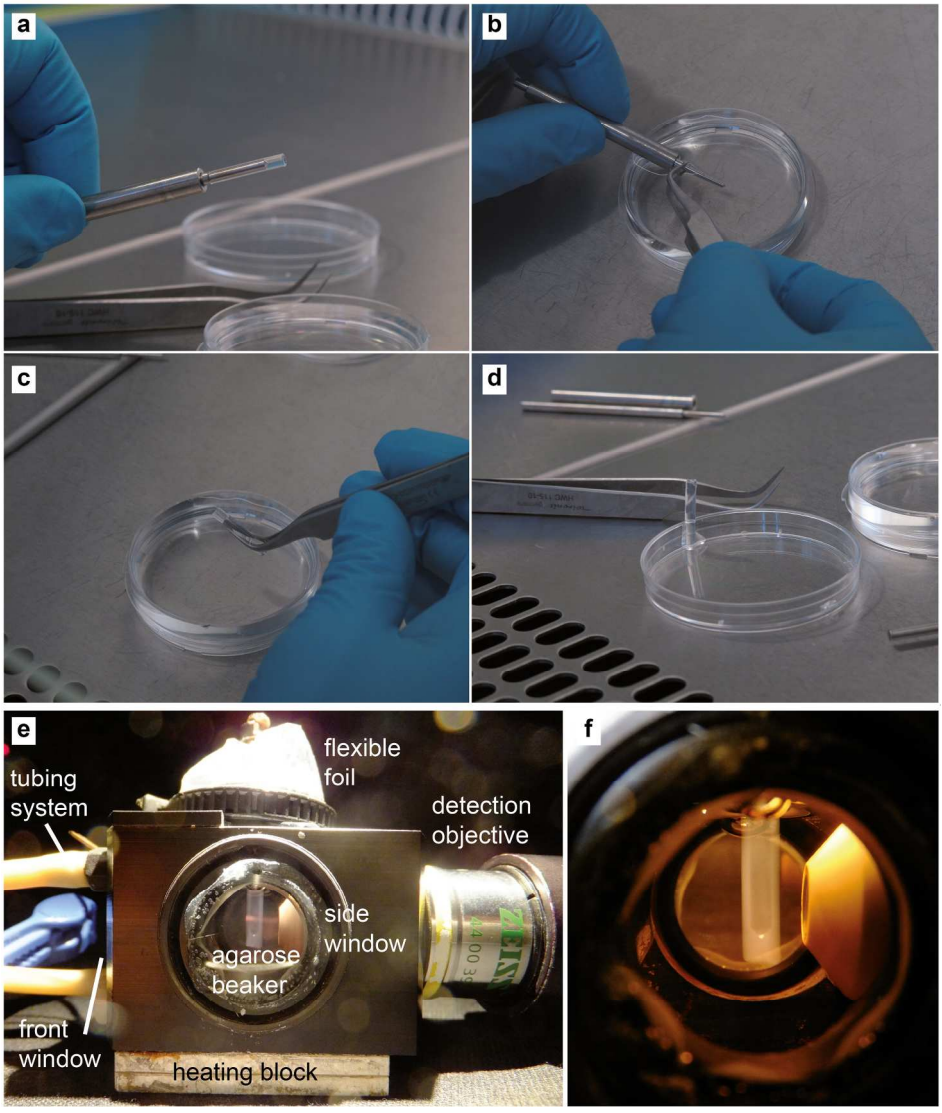
Supplementary Figure SF2



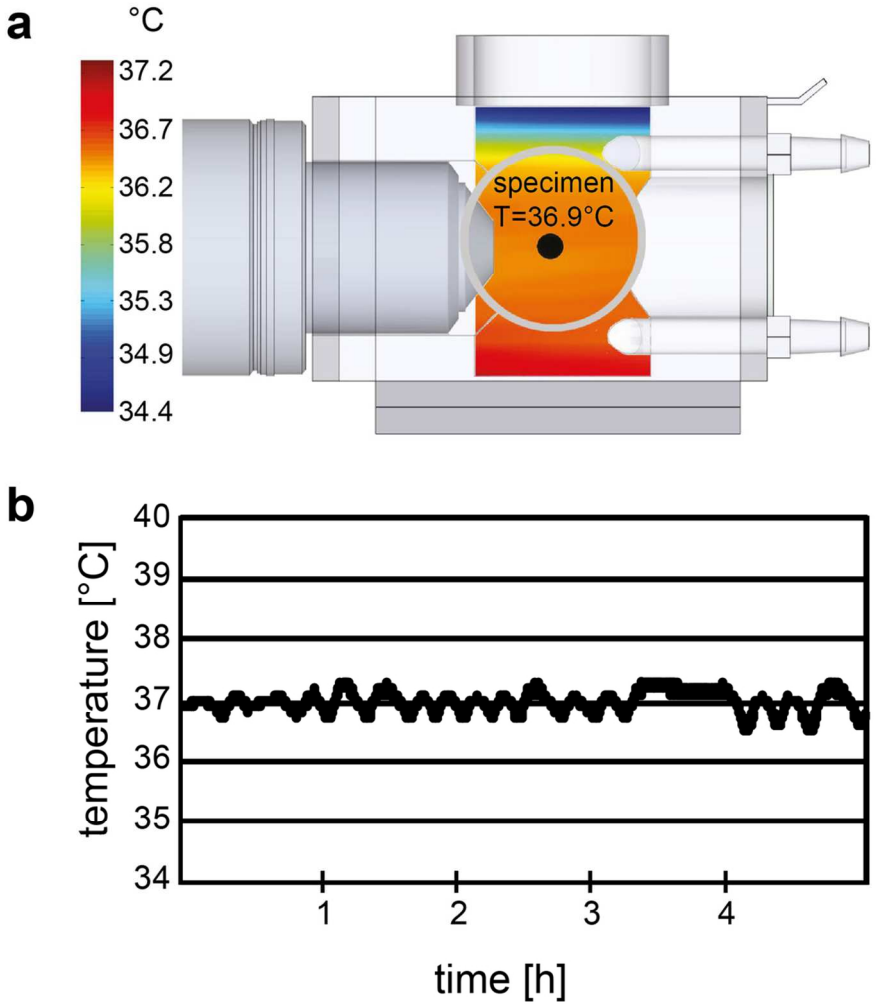
Supplementary Figure SF3



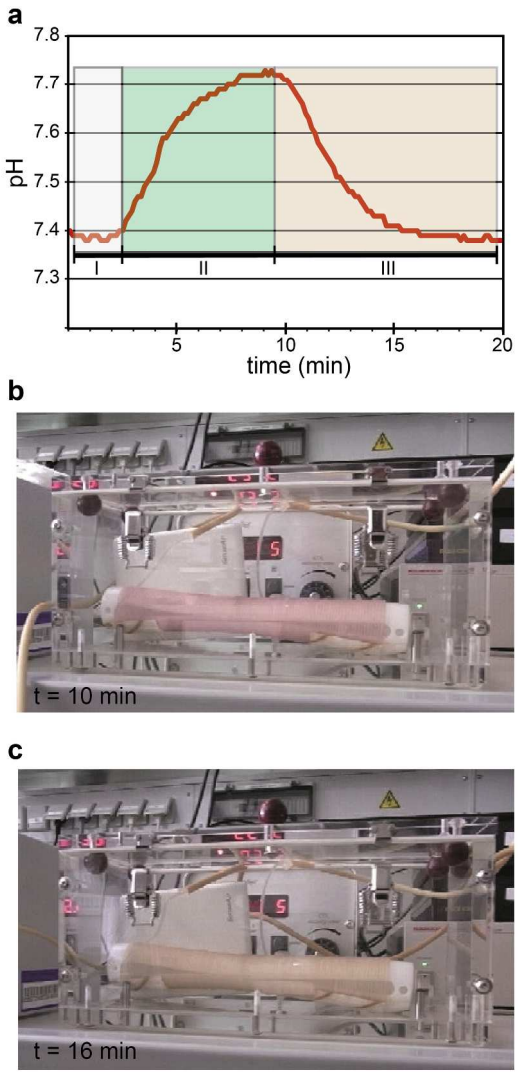
Supplementary Figure SF4



Supplementary Figure SF5



Supplementary Figure SF6



Supplementary Figure SF7