SUPPLEMENTARY METHODS

Sample preparation
For SPIM, paper mulberry pollen grains (Polysciences Inc., Warrington, PA, USA) were embedded in 1% low melting agarose gel. For confocal imaging they were embedded in Mowiol. MDCK cells stably expressing GFP-actin (a gift from G. Fenteany, University of Illinois) were cultured for several days in a three-dimensional collagen I matrix. After cyst development, a small fragment was cut from the sample, incubated for 10 minutes in 0.5 µM DRAQ5 (Alexis Biochemicals) in medium, and embedded in agarose. A 100 µl glass micropipette (Brand GmbH, Germany) was employed to extract the sample. After hardening, the sample was extracted from the capillary, and mounted in the SPIM for imaging, as described by Greger et al. Cellular spheroids of BxPC3 human pancreatic cancer cells (about 800 cells each) were prepared using the hanging drop method. The spheroids were mixed with low gelling temperature agarose dissolved in PBS pH 7.4 (Sigma-Aldrich A-4018, concentration 0.5% w/vol). Single spheroids were extracted as above for MDCK cysts, incubated for 15 minutes in 0.1 µM DRAQ5, and mounted in the SPIM for imaging.

Single Plane Illumination Microscopy
SPIM was performed as described by Huiskens et al. and Greger et al., employing a Hamamatsu Orca-ER camera with a pixel-pitch of 6.45 µm. For the pollen grain data, a 63×/0.9 NA water immersion objective was used (yielding a 100 nm pixel size). The excitation was at 488 nm, and the fluorescence emission was detected through a long-pass filter (> 510 nm). The data were acquired along 36 different directions, rotating the sample over 360 degrees. Total acquisition time was approximately 15 minutes. For the MDCK cyst data, a 40×/0.8 NA water immersion lens was used (yielding a 160 nm pixel size). The excitation of GFP-actin was at 488 nm and the fluorescence was detected through a long-pass filter (> 500 nm). DRAQ5 was excited at 633 nm and detected with a long-pass filter (> 650 nm). Six views of the MDCK cyst were recorded, rotating the sample over 180 degrees. Acquisition time was approximately 15 minutes. For the pancreatic tumor spheroids, a 40×/0.8 NA water immersion lens was used (yielding a 160 nm pixel size). DRAQ5 was excited at 633 nm, and detected with a long-pass filter (> 650 nm). Twelve views of the spheroid were recorded, rotating the sample over 360 degrees. Acquisition time was approximately 30 minutes.

Confocal microscopy
For confocal microscopy of the pollen grains and the MDCK samples, a Leica TCS SP2 AOBs system (Leica, Wetzlar, Germany) was used. Pollen grain data were recorded using a 63×/1.4 NA oil immersion objective. The excitation wavelength was 488 nm, and the fluorescence was detected between 510 and 600 nm. For the MDCK cyst data, a 63×/1.2 NA water immersion objective was used. The excitation of GFP-actin was at 488 nm, and the fluorescence was detected between 500 nm and 550 nm. DRAQ5 was excited at 633 nm, and was detected between 650 nm and 750 nm. Acquisition time was approximately 45 minutes.

Measurement of Point-Spread-Functions
Point-Spread-Functions (PSF) were derived from multiple images of 200 nm beads that were acquired under conditions identical to those used in the experiments. The
PSF was calculated from the bead images using deconvolution with an appropriate bead model as described in the literature\(^5\).

**SPIM data processing and deconvolution**

To obtain registered stacks with the same orientation and isotropic sampling, preprocessing was done as described by Huisken et al.\(^4\) The weighted spectral averaging reconstructions were done as described by Swoger et al.\(^6\). Deconvolution was done using measured PSFs by a modification of an algorithm that was described by Verveer et al.\(^7\). The object \(f\) is estimated by minimizing the following criterion:

\[
\Phi = \sum_{i=1}^{N} \left\| W (H_i f - g_i) \right\|^2
\]

where \(N\) is the number of data sets that are simultaneously deconvolved. The vector \(f\) is formed by concatenating the voxel values of the 3D object that is estimated, \(g_i\) is a vector obtained by concatenating the values of the stack from the \(i\)th rotation, and the \(H_i\) are blockcirculant matrices derived from the PSFs for each data set. Matrix-multiplication of \(f\) with \(H_i\) is equivalent to convolving the 3D image represented by \(f\) with the \(i\)th PSF, which was implemented using fast Fourier transforms. The \(\| \cdot \|\) operator represents the Euclidean vector norm. \(W\) is a weighting matrix, which in our case was implemented by a Gaussian smoothing filter with a standard deviation equal to one pixel. The objective function was minimized by a conjugate gradient algorithm after substituting the transformation \(f = x^2\) to implement a non-negativity constraint. The solution is regularized by the weighting matrix \(W\) and by limiting the number of iterations of the conjugate gradient algorithm. The algorithm was implemented in the Python programming language with the Numarray extension for numerical computation. The code is licensed under the GNU General Public License (GPL) and available on request from the authors. Since the data sets were too large to be processed by a single computer they were divided in parts, processed on a computer cluster equipped with 2.4 Ghz dual-Pentium 4 processors, and then recombined into a single image stack. Processing time for a single part was 1-2 hours, yielding total processing times up to 12 hours. Effective processing time was however equal to that for a single part, due to parallel processing of the parts on the computer cluster.

**Confocal data processing and deconvolution**

Confocal data were resampled to obtain isotropic sampling and deconvolved using measured PSFs. The same algorithm was used as for the SPIM data, by setting the number of data sets \(N = 1\). Deconvolution with a standard algorithm that is optimized for low-intensity images distorted by Poisson noise did not provide improved results.

**References**