Supplementary Information:

Marker-Free Detection of Progenitor Cell Differentiation by Analysis of Brownian Motion in Micro-Wells

Optimization of micro-well dimension

We chose the diameter of micro-wells in order to minimize double occupancy of micro-well and at the same time maximize space for free diffusion inside the well. A set of wells with diameters of 15 to 50 was examined. We concluded that 35 µm diameters of the well is optimal for GMP cell line. In these micro-wells, cells showed 90% viability and 60% occupancy of single-cell per well. Given an average diameter of 16 for GMPs the clearance between cell diameter and well diameter (L) is 19 µm.

Mean square displacement (MSD)

Mean square displacement is commonly used to characterize random motion of a particle and the diffusion coefficient and confinement. In our setup, there is no flow and gravitational forces cancel random motion in z direction in our time-intervals. In our analysis, we have decoupled the z-motion from the random motion in x and y, while, cells exhibit an almost constant distance from the substrate at all time. Hence, as in many other studies on colloids at the surface, we can treat the x-y motion as effective 2D diffusion. The MSD for free diffusion in 2D is given by:

\[
<|\Delta r|^2> = 4Dt
\]

SI.Eq.1

Confinement affects the diffusion of particle\(^1\), we have used the MSD equation derived by Bickel\(^2\) for 2D diffusion in circular domain. Since the diameter of cell is comparable to diameter of micro-well, we have substituted the diameter of micro-well with the clearance L (Fig.SI.1):

\[
<|\Delta r|^2> = \frac{L^2}{4} \left( 1 - 8 \sum_{m=1}^{\infty} \frac{\alpha_{2m}^2}{\alpha_{1m}^2 (\alpha_{1m}^2 - 1)} \exp \left\{ -4\alpha_{1m}^2 \frac{Dt}{L^2} \right\} \right)
\]

SI.Eq.2

Here \( \alpha_{nm}^2 > 0 \) \( m \)th root of Bessel prime function, \( J'_{n}(\alpha_{nm})=0 \). We have used the first two expression of summation and fitted the MSD values from experiment with the equation SI.Eq.2. D is the free diffusion coefficient, from Einstein relation for spheres:

\[
D = \frac{K_b T}{6\pi \eta r}
\]

SI.Eq.3

assuming T=37 °C and approximating viscosity of medium with water at 37 °C, \( \eta = 0.7225 \text{mPa.s} \) and average radius of cell \( r=8\mu m \), we derive the diffusion constant: \( D = 0.04(\mu m^2/s) \).

Image acquisition

The image acquisition was performed with 10x objective on Zeiss Axiovert 100M microscope equipped with a sensicam (PCO imaging) camera. The configuration has a resolution of 0.65 µm/pixel. Image acquisition was done in a multi-position mode collecting 120 fields of views in sequence using macros within the image software MicroManager\(^4\). A complete scan takes 3 minutes. Hence in the
time-lapse series cells typically exhibit an average displacement of 3 µm (5 pixels) within the scanning time intervals (from SI.Eq.2). The setting is good compromise between acquisition of a large number of cell data and a reasonable time resolution for tracking individual cells inside micro-wells.

To facilitate image processing, we have adopted a technique by Buggenthin et al.\textsuperscript{5} and used out of 5 focus images. While cells show a bright halo in the periphery when imaged in the focal plane by phase-contrast microscopy, in an out of focus image cells exhibit a bright center spot.

The microscope setup and heating system was turned on for at least 2 hours prior to experiment to equilibrate the temperature which minimizes the z drift. We observed a 1-2 µm z-shift over 3 days experiment which does not affect the image quality and cell-tracking.

10 Image processing

For cell tracking and image analysis, we have developed an in-house Java-based plug-in for ImageJ called MicroWellAnalysis (MWA). First module of MWA identifies the micro-well array, intensity threshold and micro-well size are given as inputs and software fit a smooth circle to the boundaries of micro-well. The second module automatically detects the wells with only single cell inside. An "Image stabilizer", a freely available plug-in\textsuperscript{6} of ImageJ\textsuperscript{7} software, corrects for scanning positioning errors by realigning all frames with respect to the micro-well pattern. In these wells, using a threshold value and average cell size, software tracks the position of the center of the cell with respect to the center of the well. The cell-recognition algorithm has an online threshold-value update and circularity check parameter to eliminate temporary changes in phase-contrast signal due to small morphological changes in cell. Trajectories of cells is exported into a customized Matlab code for change point analysis.

Change point analysis

Two separate methods were used to automatically detect the time point when cell change from non-adherent state to adherent. The displacement of cells by diffusion is analyzed by either Local standard deviation or a CUSUM analysis in order to determine the change point.

25 1- Local standard deviation

In our data-set, local standard deviation (\(\sigma_t\)) indicates the dispersion of displacement values for a defined number of steps (t) and is calculated from SI.Eq.4:

\[
\sigma_t = \sqrt{ \frac{1}{t-1} \sum_{N}^{N+t} (\Delta R_i - \overline{\Delta R}_t)^2 }
\]  
SI.Eq.4

where N is the N\textsuperscript{th} data point and \(\overline{\Delta R}_t\) is the local average of selected displacement (\(\Delta R\)) values from N to N+t. We have selected an arbitrary number of 5 data-points to automatically find change point in the displacement data. In non-adherent state, cells have more freedom in motion, hence their displacement values have higher deviation from mean, while in adherent state this deviation is much smaller.

Threshold value is defined for each experiment separately by user. It is possible that \(\sigma_t\) drops temporarily below the threshold before the actual transition point. To discard these points, we introduced persistency criterion which checks if values below the threshold stays below the threshold
for specific number of steps. If the algorithm finds more than one transition point, it automatically increases the persistency steps. The point with longest persistence crossover of $\sigma_5$ from a threshold value to a lower value is reported as the transition point.

2- Cusum

5 Cusum$^8$ indicates the deviation of data from a global target value. In our data-set cusum cumulatively sums the difference of displacement of cell and its time-average, SI.Eq.5.

\[
\text{cusum}_i = (\Delta R - \Delta R) + \text{cusum}_{i-1} \quad \text{SI.Eq.5}
\]

Displacement $\Delta R$ is the distance cell moves at each time-interval. The global time-average of displacement $\overline{\Delta R}$ was chosen as the reference value. Each state of motion has distinct distance from average value resulting in a constant slope for each state. A partial linear fit was fitted to the cusum data and all the changes in slopes were flagged. We select the prominent change in slope as the change point.

The cusum outcome confirmed that of $\sigma_5$ in most cases, and where discrepancies arose, the final decision was made manually by the user.

![Fig.SI.1. Schematic of mean square displacement of a spherical particle confined inside a circulars domain. The red line is the fit to short time-scale values indicating the free diffusion coefficient and the blue line indicates the plateau value.](image)

References of supplementary information