Supplementary Figure 1:

Using rat lymph nodes the optimum tissue dissociation method was investigated. We found that while mechanical methods including using manual tissue homogenizer and a wire mesh resulted in lowest viability compared to enzymatic (Collagenase IV) methods, on average they resulted in highest cell recovery. Therefore, mechanical dissociation with a tissue homogenizer was utilized in this study.

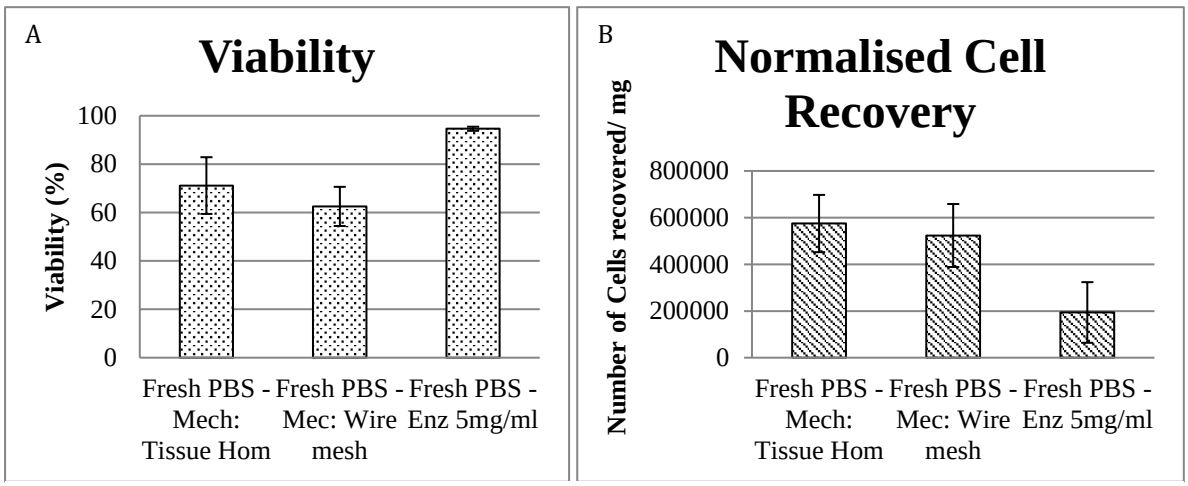

Figure S1. Viability as determined by trypan blue exclusion (A) and cell recovery (B; normalized to lymph node size) for Rat lymph node tissues stored in PBS and mechanically dissociated (Tissue homogenizer or wire mesh) or Enzymatically dissociated with 5mg/mL Collagenase IV.

Supplementary Figure 2:

To investigate cancer cell recovery for different number of cancer cells, rat lymph nodes were spiked with either 500, 10,000 or 50,000 MCF-7 cells. In each case the percent recovery was ~20% (20.15, 15.8 and 19.1%, respectively).

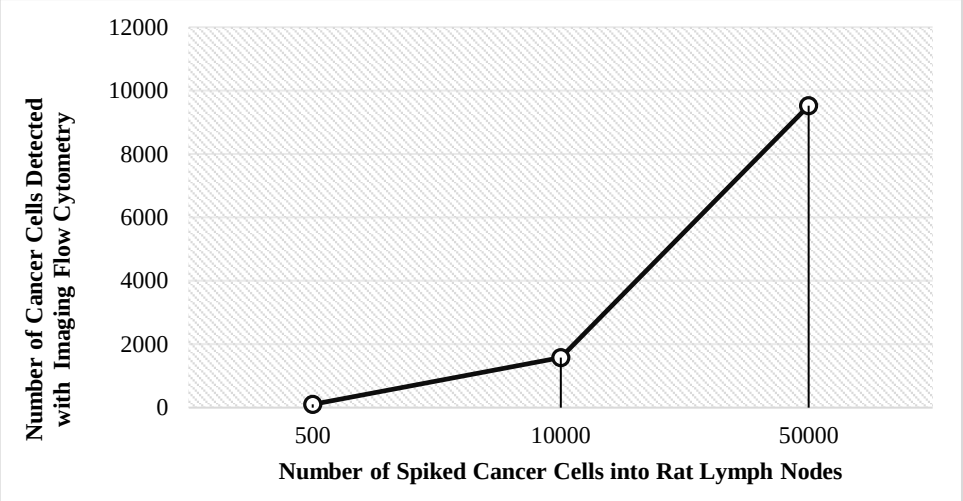

Figure S2. Number of cancer cells detected with imaging flow cytometry after lymph node spiking, homogenization and staining. Rat lymph nodes were spiked with either 500, 10,000 or 50,000 MCF-7 cells and the percent recovery was 20.1, 15.8 and 19.8%, respectively.

Supplementary Figure 3:

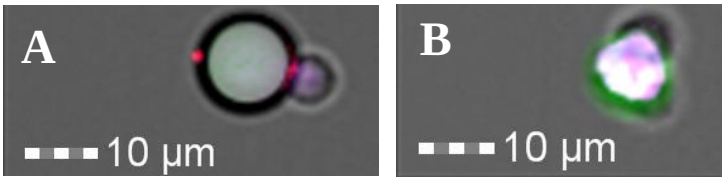

Figure S3: Examples of fluorescent bubbles and auto-fluorescent debris.

Supplementary Figure 4:

The initial gating strategy followed the Begin Analysis Wizard within the IDEAS software. Briefly, only the events/cells which were in focus were selected based on their gradient RMS (A, R1) score (based on brightfield; this measures the sharpness of an image by detecting large changes in pixel values). A scatter plot of the brightfield Area versus Aspect Ratio was then used (B) in order to select the area with cells, in this instance singles, doublets and small clumps were included for further analysis (in order not to exclude any metastatic tumour cells from enumeration). Note: in order to reduce the number of recorded events, for human samples this gate was used during the acquisition within the INSPIRE software. Next the intensity of Darkfield (Ch6) versus intensity of DAPI (Ch1) was graphed in order to select all DAPI positive nucleated cells (C) and eliminate debris without nuclear staining. A scatterplot of CD45 intensity against KRT intensity (Figure 3, D) was then used to select the 4 potential populations of interest (CD45 Positive, KRT negative; CD45 positive, KRT positive; CD45 negative, KRT negative, KRT negative and CD45 negative, KRT positive). These population gates were placed based on single cell type population fluorescence stained with all markers (CD45, KRT and DAPI).

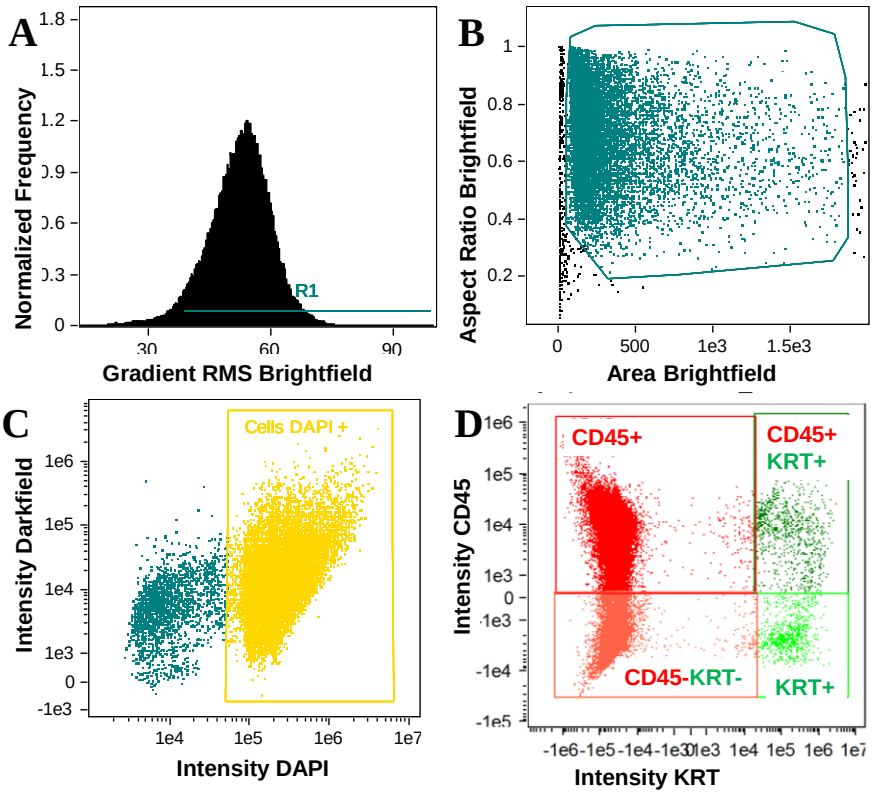

Figure S4. Gating strategy for the identification of cytokeratin (KRT) positive metastatic tumor cells in lymph nodes. (A) Histogram of Gradient RMS used for selecting in focus events. (B) Brightfield Aspect Ratio against Brightfield Area used for selecting cells and removing large clumps. (C) Intensity of Darkfield against Intensity of DAPI, used to eliminate debris and select nucleated (DAPI) positive events. (D) CD45 intensity against Cytokeratin (KRT) intensity, separating White blood cells (WBC; CD45+) from Cancerous Cells (KRT+), from double positive events (CD45+, KRT+), and double negative events (CD45-, KRT-) found with dissociated human lymph node tissue.

Supplementary Table 1:

Table S1. Compensation Table for Human Lymph Node Samples

	Ch1	**Ch2**	**Ch3**	**Ch4**	**Ch5**	**Ch6**
Ch1	1	0.082	0.076	0.025	0.055	0
Ch2	0.771	1	0.239	0.026	0.26	0
Ch3	0.238	0.211	1	0.029	0.193	0
Ch4	0.173	0.081	0.762	1	0.243	0
Ch5	0.071	0.016	0.161	0.03	1	0
Ch6	0.058	0.028	0.071	0.035	0.165	1

Supplementary Table 2:

Table S2. Compensation Table for Rat Lymph Node

	Ch1	**Ch2**	**Ch3**	**Ch4**	**Ch5**	**Ch6**
Ch1	1	0.129	0.088	0.024	0.033	0
Ch2	0.478	1	0.273	0.026	0.043	0
Ch3	0.121	0.183	1	0.029	0.035	0
Ch4	0.045	0.047	0.429	1	0.033	0
Ch5	0.03	0.016	0.175	0.03	1	0
Ch6	0.044	0.028	0.063	0.035	0.139	1