Supplementary Materials and Methods

Reagents

RPMI medium, FBS, HEPES buffer solution, sodium pyruvate, penicillin, and streptomycin were obtained from Biological Industries (Kibbutz Beit-Haemek, Israel). Rhodamine123, and formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Giemsa was obtained from Diagnostica Merk (Darmstadt, Germany). Fluorescein diacetate (FDA) was obtained from Riedle-de Haen AG (Seelze, Germany). Working FDA solutions (0.6-3.6 μM in PBS) were prepared from 25 mM stock solution in DMSO, stored at room temperature for one week or less. Anti-human anti-Bcl-2 monoclonal antibodies were purchased from Oncogene Research Products (Darmstadt, Germany). Anti-mouse fluorescein conjugated IgG antibodies were obtained from Chemicon International (Temecula, CA). Ficoll Paque 400 was obtained from Pharmacia LKB (Upsala, Sweden).

Peripheral blood lymphocytes (PBL) isolation

PBL were isolated from heparinized blood (10ml) by the standard cell density Ficoll Paque gradient. Following separation, cells were suspended in complete RPMI medium at $2x10^6$ cells/ml. In all instances, more than 70% of the cells were defined as T lymphocytes (CD3 positive). Viability, determined by propidium iodide (PI) staining, was always higher than 90%.

Cell culture

U937 promonocytic cell lines and Jurkat human T-lymphoblast cell lines were grown in a humidified atmosphere containing 5% CO_2, in RPMI 1640 medium, supplemented with 10% (v/v) heat inactivated fetal calf serum, 2 mM L-glutamine, 10 mM Hepes buffer solution, 1 mM Sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin. Molt-4 cells were grown in a humidified atmosphere containing 5% CO_2, in RPMI 1640 medium, supplemented with 10% (v/v) heat inactivated fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Intracellular enzyme activity in individual cells

Jurkat T cells ($3.5*10^6$/ml) were settled within the CR, and an initial image was taken to determine the baseline background noise. At time point zero, 10µl of 0.6µM FDA solution in PBS was applied to the localized cells, and the same field was sequentially imaged six times. The same procedure was repeated for all the other substrate concentrations (1.2, 2.4, and 3.6 µM FDA). This yielded 6 FI data points for each cell, for each dye concentration, from which the formation rate of the product was extracted, and the individual cells' Michaelis-Menten Km and Vmax values were calculated. At the end of the measurement, the cells were washed twice with a fresh buffer, and a PI solution (2.5µg/ml) was added for 5 min. The cells were then washed again, and another image was taken. PI positive cells were excluded from the kinetic analysis.

Giemsa staining

For cell fixation within the CR, 10µl of formaldehyde (4%) was added, and the cells were incubated at RT for 10 min. After washing with PBS, 10µl of fresh Giemsa solution (1:3 in water, v/v) was added and the cells were incubated for 15-20 min at RT. Cells were then washed twice with water.

Osmolytic cell bursting

Jurkat T cells ($3.5*10^6$/ml) were settled within the CR, and an initial image was taken. Then, 10µl of hypo-osmotic phosphate buffer solution (pH 7.5) was added, and the cells were incubated at RT for 10 min. After washing with phosphate buffer, PI containing solution (2.5µg/ml) was added for 5 min, followed by another wash.

Intracellular protein staining

Cells settled within the CR were fixed with 4% formaldehyde (10 µl) for 10 min at RT, washed with PBS and incubated in cold 50% methanol at 4ºC for 10 min. After additional washing with PBS, cells were permeabilized for 5 min at RT with 0.1% saponin, washed again with PBS and incubated

with 1% FCS in PBS for 15 min at RT. Cells were washed again, and anti-Bcl-2 mAb or anti-Bax mAb were added at RT for 45 min. Cells were then washed and incubated with a second FITC/Cy - conjugated anti-mouse IgG at RT, washed again with PBS and imaged.

Scanning Electron Microscopy

Jurkat T cells were introduced into the CR at an effective concentration of $3.5*10^6$ cells/ml. Cells were cultured in RPMI 1640 medium with no additions. For SEM observations, cells were fixed with 2.5% glutaraldehyde, washed with PBS without Ca and Mg, and washed again with the same PBS buffer. For gradual dehydration, cells were sequentially washed with increasing concentrations of alcohol (30-100%). Then, cells were carefully treated with Freon at 20-100%. Cells in the CR were sputter-coated with gold (up to 10 nm), and studied using Jeol JSM-840 scanning electron microscope (Tokyo, Japan) at an operating voltage of 25 kV.

Supplementary Figure and Movie Legends

Supplementary Figure Legends

Supplementary Figure S1. By applying blind deconvolution to diffusive-convoluted bright-field (**A**) and fluorescent (**C**) images, an impressive enhancement was obtained, as shown in images (**B**) and (**D**) respectively (here a set of R123-stained Molt 4 cells is shown).

Supplementary Figure S2. Osmolysis of Jurkat T cells within the PWs. Intact cells (**A**) were first stained with PI (0.5 μg/ml) to exclude dead cells – the right-hand bright-red cell in (**B**) is PI-positive (dead). Next, the cell membrane was removed by applying a hypotonic solution, causing the cells to swell (**C**) terminating in osmolytic bursting (**D**). Then, the ACR was re-rinsed with PI, staining the intact nuclei, each preserved in its original PW (**E**).

Supplementary Figure S3. (**A**) A scattergram of FI versus FP of FDA-stained peripheral lymphocytes. Following in situ permeabilization of the same cells, intracellular fluorescein is released. Consequently the FI could not be detected by the camera, thus only yielding background signals. (**B**). Then, the sample was incubated with anti-Bcl-2 mAb, followed by FITC-conjugated anti-mouse IgG, and the fluorescence was measured again, showing the frequency of Bcl-2 protein expression (**C**). Finally, intracellular esterase activity on FDA substrate and Bcl-2 protein expression are correlated at a single cell resolution (**D**).

Supplementary Movie Legends

Supplementary Movie S1. Free movement of non-adherent non-tethered Molt cells retained within their individual picowells.

Supplementary Movie S2. The ACR – schematic demonstration of construction, cell loading and bio-manipulation.