The Lifeact-EGFP Mouse is a Translationally Controlled Fluorescent Reporter of T Cell Activation

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SUPPLEMENTARY INFORMATION

SUPPLEMENTARY FIGURES

Fig. S1

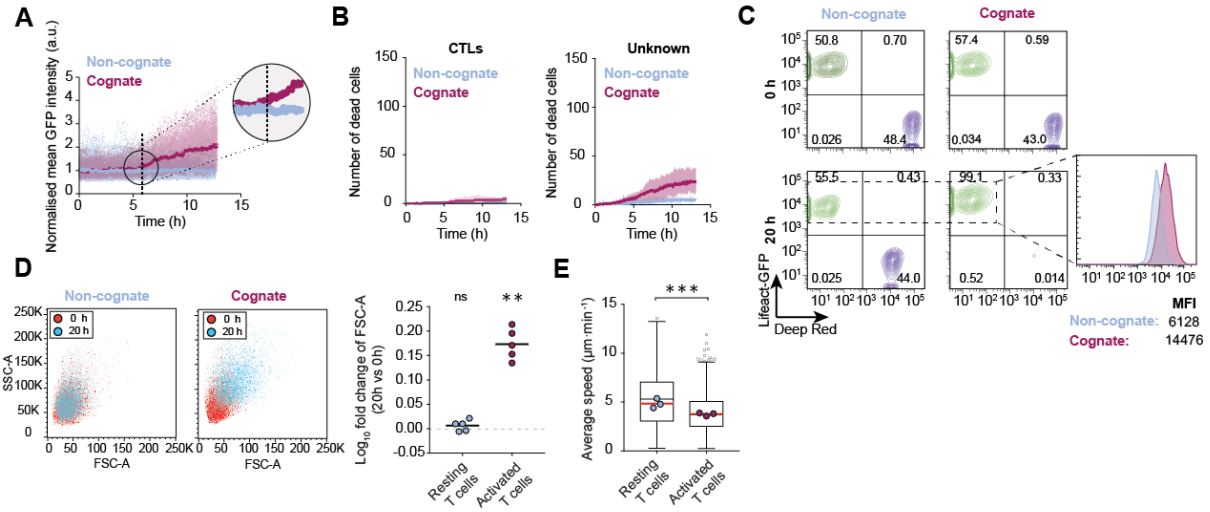


Figure S1. Determining time of activation, size and granularity in LGO1 cell populations.

A Time-series of normalised distributions of mean GFP intensities of LGO1 cells interacting with cognate (n=722 T cells, red) or non-cognate (n=650 T cells, blue) EL-4. Medians per timepoint shown as thick lines. Time of divergence/activation determined by means of a non-parametric right-tailed Wilcoxon rank sum test ($p < 0.0014$), indicated by dashed black line.

B Number of tracked PI surfaces ascribed to LGO1 effector CTLs (left) or that could not be ascribed (right) when LGO1 were co-embedded in a 1:1 ratio with cognate (red) or non-cognate (blue) EL-4 tumours in 3D collagen matrices as describes in Fig. 1E. Data points indicate the mean of pooled data from 3 independent experiments; error bars indicate 95% confidence interval.

C Flow cytometric analysis depicting intensities of Lifeact-GFP (green) in LGO1 cells and CellTracker Deep Red-stained EL-4 tumour cells (purple) in non-cognate (left) and cognate (right) contexts at 0h (top) and following 20 h of co-culture (bottom). Histogram overlays illustrates the shift in GFP fluorescence intensity when LGO1 cells are incubated with non-cognate (blue) and cognate (red) EL-4 cells.

D **Left and Middle:** Forward (FSC-A) and side (SSC-A) scatter plots of LGO1 cells incubated with non-cognate (left) and cognate (middle) EL-4 tumour cells, at 0h and 20h of incubation as indicated.

Right: Log10 fold change in FSC-A of LGO1 cells after 20h of incubation with non-cognate (resting T cells) and cognate (activated T cells) EL-4 tumour cells relative to the FSC-A of the corresponding T cell population at 0h. Bar: mean of pooled data from 5 independent experiments (data points). ns: $p > 0.05$ and ** $p < 0.01$ by one sample T test compared with a hypothetical test value of 0 (dashed line) (right).

E Average speeds of LGO1 cells imaged and tracked in 3D collagen matrices following a 20-hour incubation with non-cognate EL-4 cells (resting T cells: n= 4393) or cognate EL-4 cells (activated T cells: n=3702). Pooled data from 3 independent experiments; data points: average for each experiment; red bar: mean; box-whiskers: medians and quartiles, with outliers outside whiskers. *** $p < 0.001$ by Mann-Whitney U test.

Fig. S2

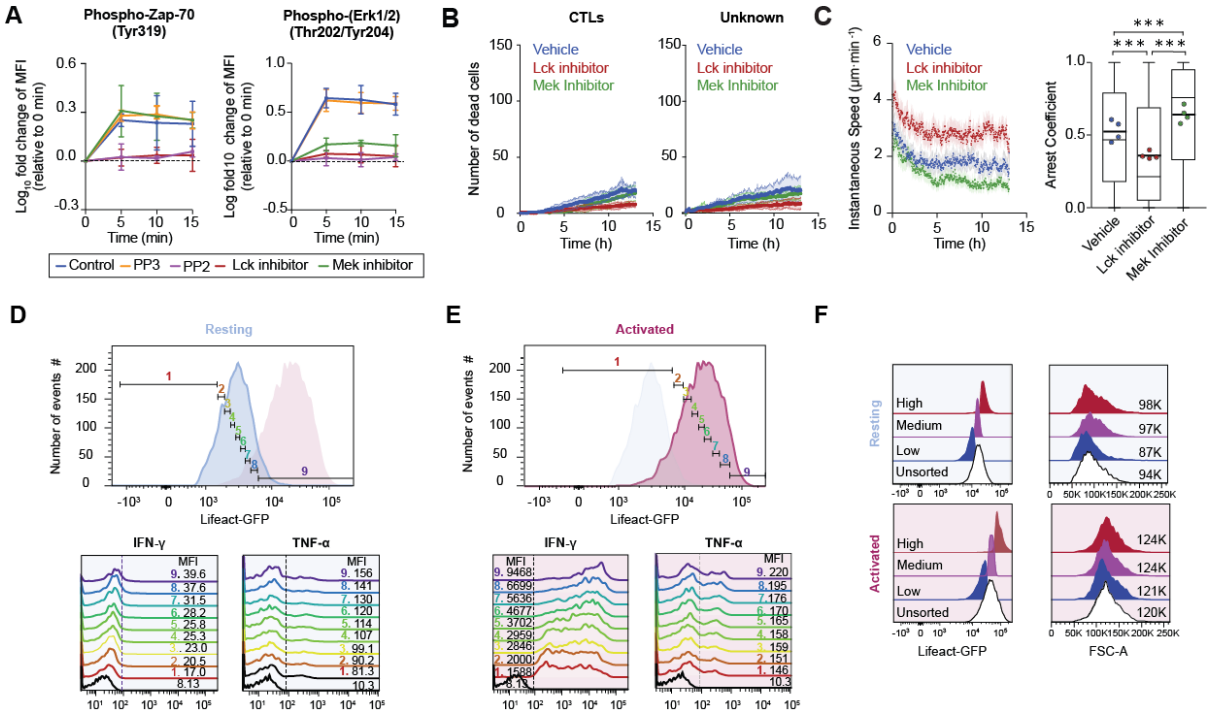


Figure S2. Lifeact-GFP enhanced fluorescent is TCR signalling mediated and it is correlated with the expression of IFN-γ and TNF-α in LGO1 cells

A Log_{10} fold change of the mean fluorescence intensity (MFI) of phospho-Zap-70 (Tyr319) (left) or phospho-Erk1/2 (Thr202/Tyr204) (right) staining intensities relative to resting values at 0 min (pre-activation) in LGO1 cells stimulated by streptavidin beads coated with cognate H-2K^b/SIINFEKL pMHC for 5, 10 and 15 min in the presence of Src-family kinase inhibitors PP2 (10μM) or PP3 (10μM), 10μM Lck inhibitor or 5μM Mek 1/2 inhibitor. Data points: mean from 3 independent experiments; error bars: range.

B Number of tracked PI surfaces ascribed to CTLs (left) or that could not be ascribed (right), when LGO1 cells were co-embedded in a 1:1 ratio with cognate EL-4 tumour targets in 3D collagen matrices treated with the indicated inhibitors or vehicle control as indicated in **Fig. 2B**. Data points indicate the mean of pooled data from 3 independent experiments; error bars indicate 95% confidence interval.

C **Left:** Instantaneous speed of LGO1 cells co-embedded with cognate EL4 tumour cells in 3D collagen matrices, treated with indicated inhibitors or vehicle control and tracked following live-cell imaging. Data points: population mean instantaneous speed from 453 vehicle, 340 Lck inhibitor and 268 Mek 1/2 inhibitor-treated T cells; error bars: 95% confidence interval; representative of 4 independent experiments.

Right: Distribution of the arrest coefficient of LGO1 cells co-embedded with cognate EL4 tumour cells and treated with inhibitors or control as indicated (Control: n=4686, Lck inhibitor: n= 4594, Mek inhibitor: n= 3666 T cells). Data points: means; box-whiskers: medians and quartiles from pooled data of 4 independent experiments; thick bars: mean of pooled data; *** $p < 0.001$ by Kruskal-Wallis test followed by Dunn's multiple comparison test.

D **Top:** Representative histograms showing the designation of 9 gates based on GFP mean fluorescence intensity in resting LGO1 cells after 20h of incubation with the non-cognate tumour cells.

Bottom: Mean fluorescence intensities (MFI) of IFN-γ (left) and TNF-α (right) for each gate as indicated for resting LGO1 cells. Representative plots from 4 independent experiments.

E **Top:** Representative histograms showing the designation of 9 gates based on GFP mean fluorescence intensity in activated LGO1 cells after 20h of incubation with the cognate tumour cells.

Bottom: Mean fluorescence intensities (MFI) of IFN-γ (left) and TNF-α (right) for each gate as indicated in activated LGO1 cells. Representative plots from 4 independent experiments.

F **Left:** Fluorescence intensity of sorted LGO1 populations distinguished by GFP intensity after co-incubation for 20h with non-cognate (resting, top) or cognate (activated, bottom) EL4 tumour cells. An unsorted population was established as a control. Representative plots from 3 independent experiments.

Right: Forward scatter area (FSC-A) histograms of LGO1 cells after cell sorting from the co-culture for 20h with non-cognate (resting, top) or cognate (activated, bottom) EL4 tumour cells. Representative plots from 3 independent experiments.

Fig. S3

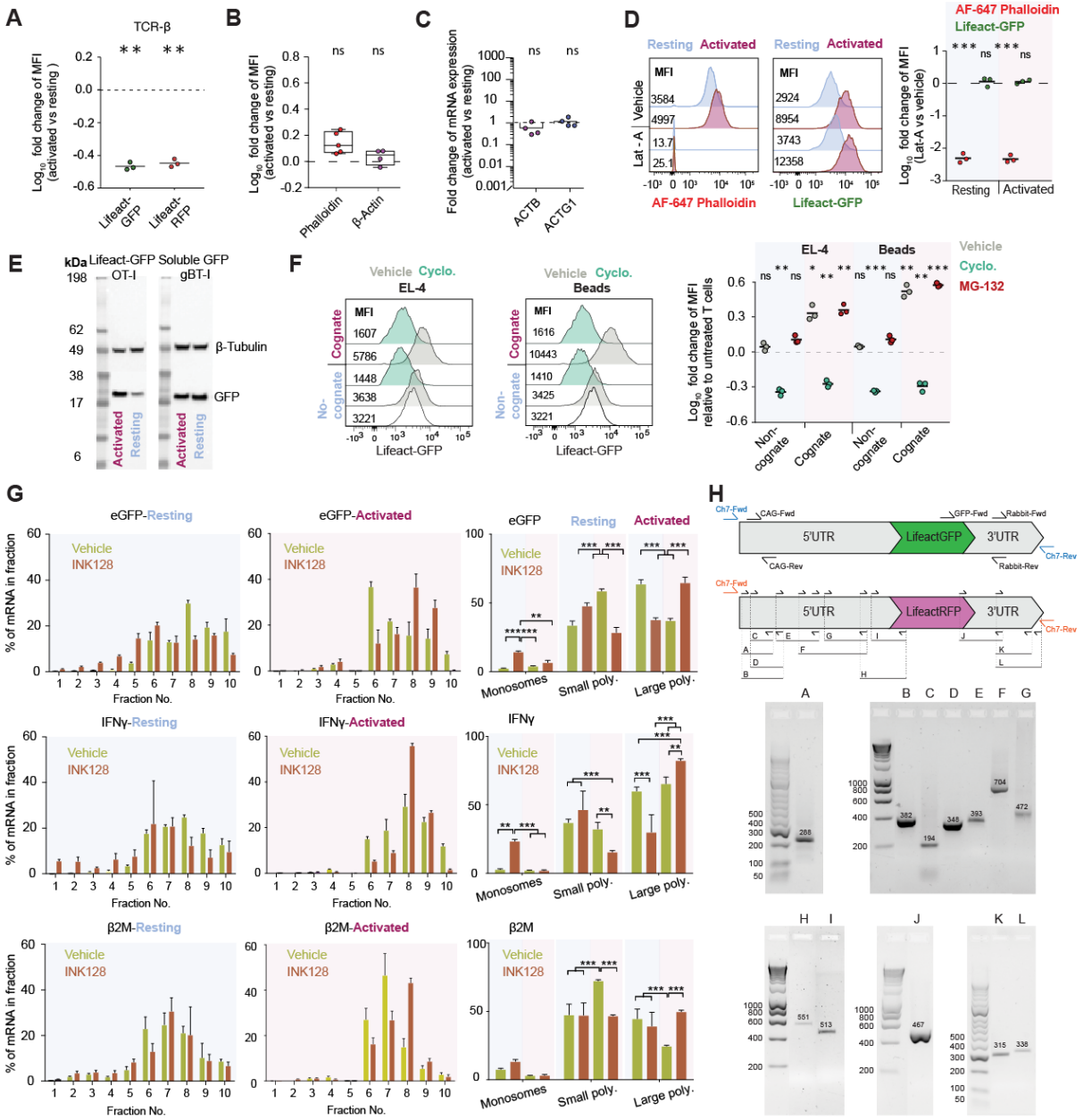


Figure S3. Gain in Lifeact-GFP fluorescence is not related to F-actin polarization and it is regulated at the translational level.

A Log_{10} fold change of the mean fluorescence intensity (MFI) of TCR-Vα expression in polyclonal effector T cells from the Lifeact-GFP or Lifeact-RFP mice stimulated for 20h with streptavidin beads coated with biotinylated CD3/CD28 antibodies (activated) or beads coated with biotinylated IgG isotype control antibodies (resting). Data points are from independent experiments, bars represent the mean. ** p <0.01 by one sample T test compared with a hypothetical test value of 0 (dashed line).

B Log_{10} fold change of the mean fluorescence intensity (MFI) of phalloidin and anti-β-actin staining in activated LGO1 cells relative to resting cells. LGO1 cells were pre-incubated

for 20h with cognate (Activated) or non-cognate (Resting) EL4 tumour cells respectively as measured by flow cytometry. Data points: 4 independent experiments; box-whiskers: medians and quartiles of the pooled set. ns: $p > 0.05$ by one sample T test compared with a hypothetical test value of 0 (dashed line).

C Fold change of mRNA expression via quantitative real-time PCR analysis of ACTB and ACTG1 in LGO1 cells sorted from cognate (activated) versus non-cognate (resting) EL4 tumour cells after 20h of incubation. Expression levels normalised to housekeeping genes RPL13A and β2M. Data points: 4 independent experiments; bars: mean. ns: $p > 0.05$ by one sample T test compared with a hypothetical test value of 0 (dashed line).

D **Left and Middle:** Representative histograms showing the mean fluorescent intensities (MFI) of phalloidin conjugated with Alexa Fluor 647 (AF-647) staining (Left) or Lifeact-eGFP (Middle) in activated or resting LGO1 cells treated with vehicle or 1μM latrunculin A (Lat-A). LGO1 cells were pre-incubated for 20h with cognate (activated) or non-cognate EL4 (resting) tumour cells.

Right: Log_{10} fold change of MFI of phalloidin staining or Lifeact-EGFP in activated or resting LGO1 cells treated with LatA compared to vehicle. Data points: 3 independent experiments; bars: mean. ns: $p > 0.05$ and *** $p < 0.001$ by one sample T test compared with a hypothetical test value of 0 (dash line).

E Western Blot showing protein levels of β-tubulin (loading control) and eGFP in sorted LGO1 or UBI-GFP.gBT-I cells after 20h of incubation with cognate (activated) or non-cognate (resting) EL4 tumour cells.

F **Left and Middle:** Representative histograms showing the mean fluorescent intensities (MFI) of Lifeact-eGFP in LGO1 cells pre-treated with cycloheximide 10μM (Cyclo.) or vehicle and then stimulated for 20h with cognate or non-cognate EL4 cells (Left) or with cognate (H-2K^b/SIINFEKL) or non-cognate (H-2K^b/RGYVYQGL) beads (Middle).

Right: Log_{10} fold change of Lifeact-GFP mean fluorescence (MFI) in LGO1 cells pre-treated with Cyclo. 10μM, MG-132 5μM or vehicle in the presence of cognate or non-cognate EL4 cells or with beads. The MFI is relative to the Lifeact-eGFP fluorescence of untreated resting LGO1 cells, as measured by flow cytometry. Data points: 3 independent experiments; bars indicate the mean of the pooled set. ns: $p > 0.05$, * $p < 0.05$, ** $p < 0.01$and *** $p < 0.001$ by one sample T test compared with a hypothetical test value of 0 (dashed line).

G Distribution of eGFP, IFNγ and B2M messages across monosomes, small and large polysomes quantified by RT-qPCR. **Left and Middle:** Percentage distribution of mRNA

expression of eGFP (Top), INF-γ (Centre) and β2 microglobulin (Bottom) for each polysome fraction in sorted LGO1 cells pre-treated with INK128 or vehicle and then incubated with non-cognate (Resting, left) or cognate (Activated, middle) EL-4 tumour cells. **Right:** Percentage distribution of mRNA expression of eGFP (Top), INF-γ (Centre) and β2 macroglobulin (Bottom) in monosomes (fractions 1-4), small polysomes (fractions 5 to 7) and large polysomes (fractions 8 to 10) from sorted LGO1 cells pre-treated with INK128 or vehicle and then incubated with cognate (activated) or non-cognate (resting) EL-4 tumour cells. Data are means ± S.D., n = 3. *: $0.01 \leq p < 0.05$; **: $0.001 \leq p < 0.01$; ***: $p < 0.001$ (Two-way ANOVA)

H Amplification and/or sequencing of flanking regions of the Lifeact-GFP (Top) and Lifeact-mRFPruby (Bottom) transgenes confirms that the 5' and 3' UTRs are identical in the two transgenic mice. Indicated primers are reported in Methods.

Fig. S4

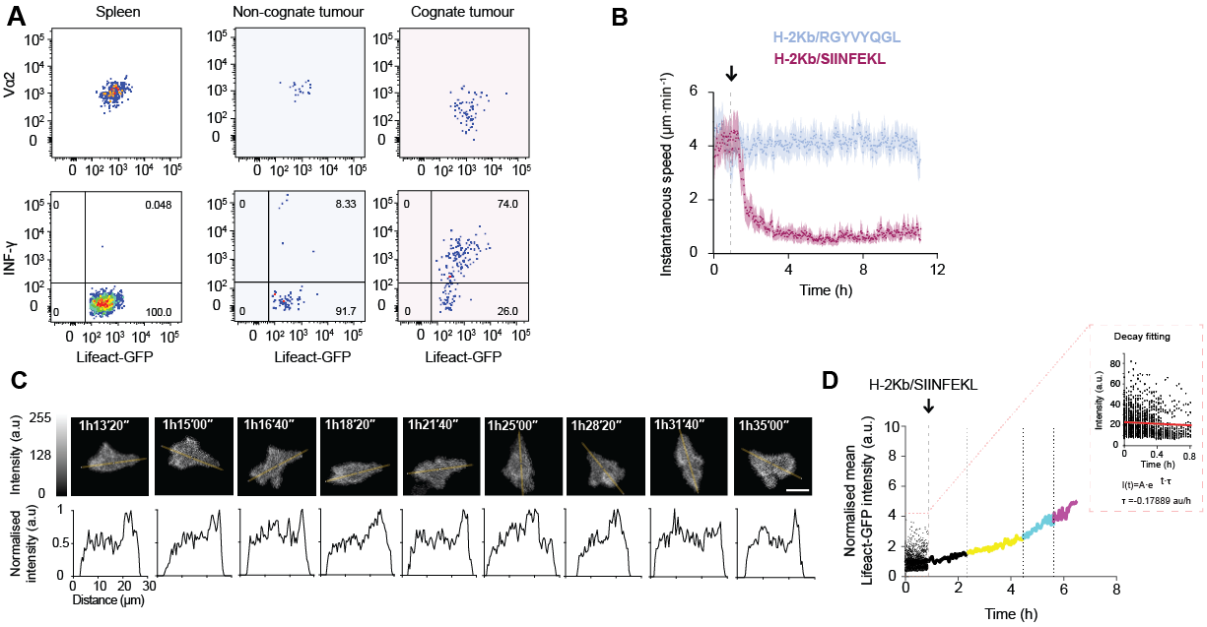


Figure S4. Enhanced Lifeact-GFP fluorescence can be monitored *in-vivo* and it is identifiable at a single-cell imaging.

A **Top:** Representative flow cytometry plots showing the expression of Lifeact-GFP and TCR-Vα2 in effector LGO1 cells isolated from spleen (left), non-cognate (centre) or cognate tumours after 24h post-T cell transfer.

Bottom: Representative flow cytometry plots showing the expression of Lifeact-GFP and IFN-γ in effector LGO1 cells isolated from spleen (left), non-cognate (centre) or cognate tumours after 24h post-T cell transfer.

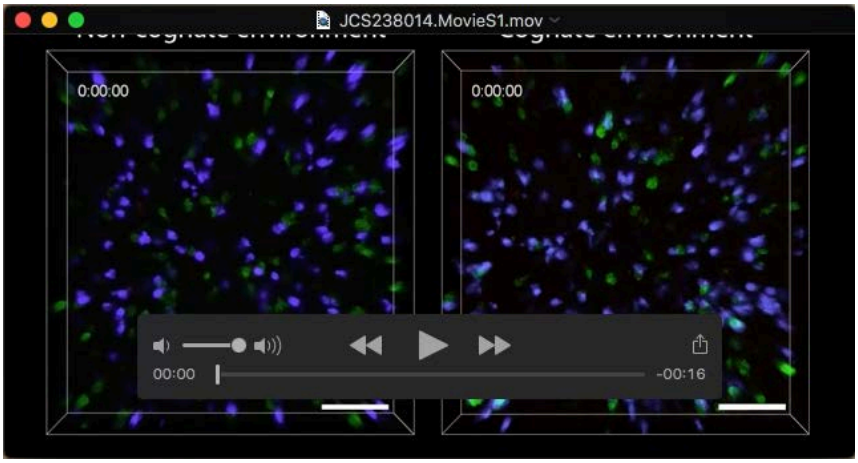
B Instantaneous speeds of LGO1 cells embedded in 3D collagen matrix. After ~1 h of imaging (dashed line) either cognate (H-2K^b/SIINFEKL, n=832 T cells) or non-cognate (H-2K^b/RGYVYQGL, n=1614 T cells) monomeric pMHC was added to the bathing medium as indicated. Data points: population mean; error bars: 95% confidence interval.

C Top: maximum intensity projections of F-actin localisation (Lifeact-EGFP) in an LGO1 cell embedded in 3D collagen matrix following acute addition of monomeric non-cognate pMHC at 0h. Orange lines: region of intensity linescan analysis (bottom).

D Main: Normalised GFP mean fluorescence intensity (MFI) before treatment (black data points, all cells) and representative cell after acute addition of cognate pMHC (dashed lined) (black, yellow, cyan, magenta solid curve). Black, yellow, cyan and magenta indicate regimes of the T cell activation confidence levels.

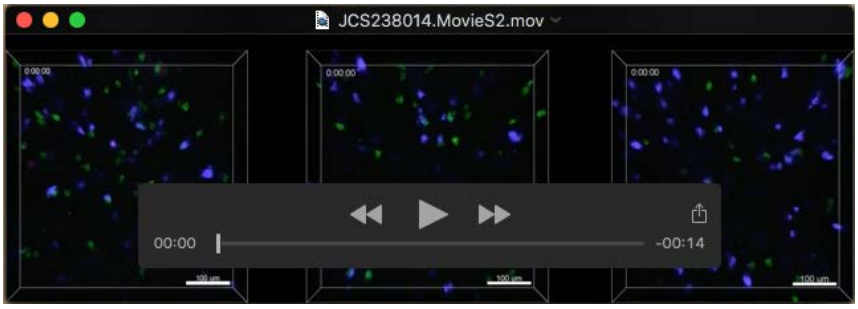
Inset: GFP intensity distributions before addition of cognate pMHC re-aligned such that all tracks initiate at 0h prior to photo-bleaching correction (black triangles). Mono-exponential decay function (red line) fitted to data set and subsequently used for normalisation of data in Main plot.

SUPPLEMENTARY MOVIES



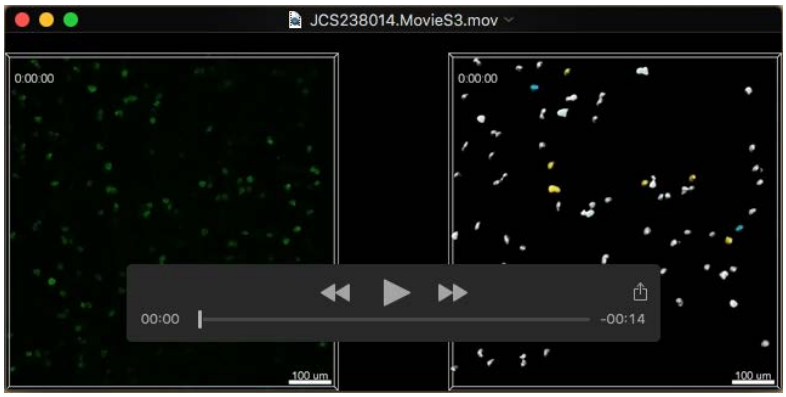
Movie 1 – Search and kill assay using cognate targets

Live-cell imaging of LGO1 cells (green) co-embedded with non-cognate (left) or cognate (right) EL-4 tumour cells (blue) in a 3D collagen matrix over 12 h. Killed cells appear in pink (propidium iodide uptake). Time in h:min:s. Scale bar: 100µm



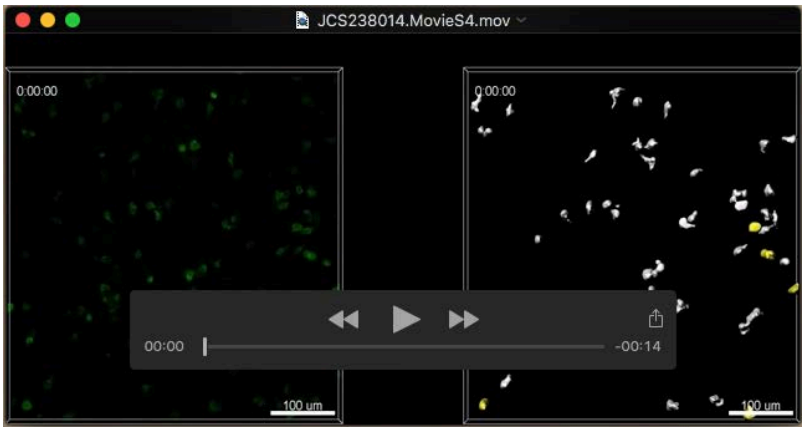
Movie 2 – Search and kill assays of cognate targets in the presence of Lck and Mek 1/2 inhibition

Live-cell imaging of LGO1 cells (green) co-embedded with cognate EL-4 tumour cells (blue) in a 3D collagen matrix over 12 h in the presence of vehicle (left), 10 µM Lck inhibitor (middle) and 5 µM Mek 1/2 inhibitor (right). Killed cells appear in pink (propidium iodide uptake). Time in h:min:s. Scale bar: 100µm



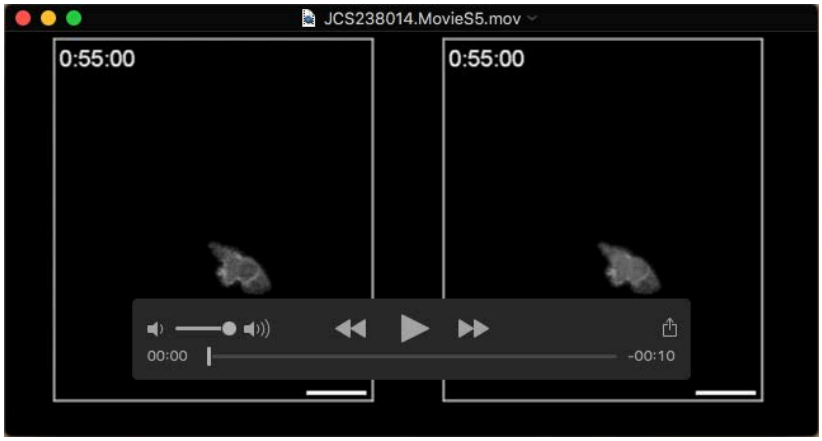
Movie 3 – Identification of activated T cells in 3D imaging data in a cognate environment

Live-cell imaging of LGO1 cells (raw fluorescence, left; coloured surface rendering, right) co-embedded with cognate EL-4 tumour cells (not shown) in a 3D collagen matrix over 12 h. Surfaces are coloured according to activation confidence level as described in **Figure 5A**: grey: indeterminate, yellow: low, cyan: medium, magenta: high. Time in h:min:s. Scale bar: 100µm



Movie 4 – Identification of T cells in 3D imaging data in a non-cognate environment

Live-cell imaging of LGO1 cells (raw fluorescence, left; coloured surface rendering, right) co-embedded with non-cognate EL-4 tumour cells (not shown) in a 3D collagen matrix over 12 h. Surfaces coloured according to activation confidence level as described in **Fig. 5A**: grey: indeterminate, yellow: low, cyan: medium, magenta: high. Time in h:min:s. Scale bar: 100µm



Movie 5 – Live analysis of single T cell activation

Live-cell imaging of an LGO1 cell (raw fluorescence, left; transparent coloured surface rendering, right) migrating in a 3D collagen matrix following acute addition of monomeric cognate pMHC molecules (t=0h). Surfaces coloured according to activation confidence level: grey: indeterminate, yellow: low, cyan: medium, magenta: high. Time in h:min:s. Scale bar: 10 µm.