

1 **Lifect-GFP alters F-actin organization, cellular morphology and biophysical**
2 **behaviour**

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1 **Abstract**

2

3 Live-imaging techniques are at the forefront of biology research to explore behaviour
4 and function from sub-cellular to whole organism scales. These methods rely on intracellular
5 fluorescent probes to label specific proteins, which are commonly assumed to only introduce
6 artefacts at concentrations far-exceeding routine use. Lifeact, a small peptide with affinity for
7 actin microfilaments has become a gold standard in live cell imaging of the cytoskeleton.
8 Nevertheless, recent reports have raised concerns on Lifeact-associated artefacts at the
9 molecular and whole organism level. We show here that Lifeact induces dose-response
10 artefacts at the cellular level, impacting stress fibre dynamics and actin cytoskeleton
11 architecture. These effects extend to the microtubule and intermediate filament networks as
12 well as the nucleus, and ultimately lead to altered subcellular localization of YAP, reduced
13 cell migration and abnormal mechanical properties. Our results suggest that reduced binding
14 of cofilin to actin filaments may be the underlying cause of the observed Lifeact-induced
15 cellular artefacts.

1 **Introduction**

2

3 Live-cell actin visualization is routinely performed and presented in a large percent of
4 cell biology research, including studies where actin or the cytoskeleton may only be
5 secondary players on the observations reported. Lifeact, a small peptide with affinity for actin
6 microfilaments¹⁻³, has become one of the gold standards in live cell imaging of actin
7 structures in particular, and overall cell morphology in general. A number of reports have
8 assessed the suitability of Lifeact as a cytoskeletal marker, focusing primarily on qualitative
9 observations of which structures are preferentially labelled by Lifeact relative to other probes
10 such as phalloidin, utrophin or actin-GFP^{4,5}. It has been recently reported that Lifeact alters
11 actin filament arrangement and dynamics in fission yeast cells⁶. Similarly, strong *in vivo*
12 Lifeact expression causes sterility in fruit flies⁷, associated with severe actin defects and
13 multiple nuclei in follicle cells. In addition, the detrimental effects of strong Lifeact expression
14 in cells appear to be linked to the specific promoter and fluorescent protein tag used^{8,9}. The
15 aforementioned studies have focused on highlighting the abnormal morphologies, dynamics
16 and overall behaviour of cells associated with strong Lifeact expression. Nevertheless, it
17 remains to be discerned whether low to mid-level expression of Lifeact results in unaltered
18 actin dynamics, or conversely if Lifeact induces broad dose-dependent effects on the actin
19 cytoskeleton. Such an understanding is still missing to better define the experimental
20 conditions under which Lifeact is to be considered a suitable probe to image actin structures.

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24 **Results**

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26 **Cell cultures transduced with Lifeact-GFP display altered morphologies**

27 In our experiments, we first performed an overnight transduction of human
28 Mesenchymal Stem Cells (hMSCs) with increasing concentrations (presented as Multiplicity
29 of Infection - MOI) of commercial adenoviral vectors delivering rAVCMV-LifeAct-
30 TagGFP2 plasmid. We transduced cells with MOI ranging from low levels (MOI 100) up to
31 the highest dose recommended by the supplier (MOI 1000). Samples were fixed 1-7 days
32 post transduction, co-stained with TRITC-phalloidin and DAPI, and subsequently imaged via
33 standard epifluorescence microscopy at 20x magnification (**Supplementary Fig. 1** and
34 **Supplementary Table 1**). When pooling together data at the population level, we found a
35 statistically significant increase in GFP intensity for experiments using higher MOIs
36 (**Supplementary Fig. 2**). Likewise, we found that GFP levels significantly changed with
37 increasing expression time, with the peak of expression occurring 5 days post transduction.

1 Surprisingly, we found comparable trends when we measured simple parameters that
2 describe cellular morphology and actin assembly, such as cell area and filamentous-actin (F-
3 actin) amount (**Supplementary Fig. 3**). These analogous temporal and concentration-
4 dependent trends observed at the population level suggested that intracellular Lifeact may
5 result in altered cellular and cytoskeletal morphology.

6 7 **Lifeact-GFP alters actin organization in a dose-response manner**

8 Traditional methods based on population averages may mask the fact that a great
9 variation exists in the uptake of plasmid or vector copy number for each cell within a
10 transduced cell culture^{10,11}. Thus, to accurately assess the dose-response effects of Lifeact
11 expression at the cellular level, we devised an alternative approach based on pooling
12 together single-cell data according to their measured Lifeact expression, irrespective of initial
13 MOI or time post-transduction. Two critical aspects of our methodological approach need to
14 be emphasised here. First, the quantification of parameters related to cytoskeleton
15 organization and cell morphology was performed using images obtained through TRITC-
16 phalloidin staining, i.e. independently of Lifeact-GFP driven fluorescence. By doing so the
17 cytoskeleton of cells with low Lifeact-GFP expression (displaying low GFP fluorescence
18 intensities, **Fig. 1b**) could be resolved with similar accuracy to those expressing larger Life-
19 GFP levels (**Fig. 1d**). Second, we took advantage of the 1:1 stoichiometry between the
20 Lifeact peptide and the GFP tag, and measured for each cell its total GFP fluorescence as a
21 surrogate indicator of Lifeact expression¹². Furthermore, we extended our previously-
22 developed image quantification pipelines^{13,14} to describe in a multiplex fashion the
23 organization of the cytoskeleton and nucleus of individual cells.

24 We constructed dose-response curves (DRC) to depict morphometric parameters as
25 a function of intracellular GFP intensity and observed clear morphological trends linking
26 increased Lifeact expression with altered cellular phenotypes (**Fig. 2 and Supplementary**
27 **Figure 4**). In particular, cells displaying the highest Lifeact expression had 10-fold larger
28 spread areas, smaller aspect ratios and a less stellate morphology (**Fig. 2**). Concurrently,
29 when assessing actin organization, Lifeact expression caused a 50-fold increase in F-actin
30 assembly (**Fig. 2d**), leading to stress fibres that were longer (**Supplementary Fig. 4 b**),
31 thicker (**Fig. 2e**) and with an increasing radial orientation (**Fig. 2f**). To verify that the effects
32 observed were associated with Lifeact rather than its fluorescent tag, we generated similar
33 DRC with cells transduced with the same promoter and a GFP tag only (**Supplementary Fig**
34 **5**). While the DRCs obtained were not so broad in terms of expression levels reached, we
35 verified that the dose-response behaviour was lost when only GFP was transduced. In
36 particular, multiple comparisons analysis (**Supplementary Fig 5**) showed that, even with the
37 highest transduction levels reached with the pCMV-EGFP there is no significant difference

1 with the lowest dosage with the same treatment. Furthermore, multiple comparisons
2 analysis also shows that, for the majority of parameters, there are significant differences
3 between cells with the highest dose of pCMV-EGFP and cells displaying similar GFP
4 fluorescence levels but transduced with the pCMV-Lifeact-Tag2. Additional experiments
5 using Lifeact-GFP recombinant protein delivered into the cellular cytoplasm using a
6 membrane fusion reagent resulted again in a dose-response behaviour that displayed
7 marked overlap with the results obtained using adenoviral transduction of Lifeact-GFP
8 (**Supplementary Fig 5**). Similar as before, for the majority of parameters, multiple
9 comparisons analysis showed that there were significant differences between cells treated
10 with the highest dose of pCMV-EGFP and cells treated with the highest doses of
11 recombinant plasmid. Conversely, there were no significant differences between cells
12 treated with the highest dosages of pCMV-Lifeact-Tag2 plasmid versus the recombinant
13 protein.

14 Of note, DRCs generated for all cytoskeletal parameters had at least two marked
15 regimes (**Fig. 2 and Supplementary Fig. 4**), namely a dose-response behaviour for low to
16 mid expression levels of Lifeact-GFP (*white background area in panels*) followed by a
17 saturation plateau at very high expression levels (*gray background area in panels*). In
18 addition, for some cytoskeletal parameters measured we could also identify a range of low
19 Lifeact-GFP expression levels for which no dose-response effect was observed (*yellow*
20 *background area in panels*). Similar dose-response trends were also obtained when NIH/3T3
21 or COS-7 cells were transduced with Lifeact-GFP vector, even though overall values for
22 parameters such as cell area or F-actin amount were different, as expected for different cell
23 types (**Supplementary Fig 6**). Altogether, these data evidences that Lifeact-GFP can have a
24 pronounced effect on cellular morphology and actin cytoskeleton organization. While at the
25 population level these effects are largely dependent on transduction conditions (MOI and
26 duration of expression), at the single cell level Lifeact-induced side effects display large
27 heterogeneity, being predominantly dependent on the amount of peptide expressed by each
28 cell.

29

30 **Lifeact-induced effects extend to other cytoskeletal networks and the nucleus**

31 Having confirmed the marked effects on whole cell morphology and stress fibre
32 architecture induced by Lifeact expression, we chose to focus on Lifeact-GFP adenoviral
33 transduction on hMSC and we next investigated cellular components with a strong link to the
34 actin cytoskeleton, such as microtubules and intermediate filaments. We limited our protocol
35 to MOI 1000 and 5 days post transduction -to maximise the range of Lifeact expression
36 levels- and replaced TRITC-phalloidin staining with antibodies against tubulin and vimentin.
37 Surprisingly, we found that increased levels of Lifeact expression were associated with a

1 build-up in the microtubule and intermediate filament networks (**Fig. 3**). Given the close
2 interconnectedness between the three cytoskeletal networks¹⁴, we hypothesise that
3 alterations in tubulin and vimentin assembly are a secondary result from the effects of Lifeact
4 on cell spread area, rather than a direct interaction between Lifeact peptides and tubulin or
5 vimentin monomers.

6 We additionally investigated if Lifeact could also influence the nucleus, since nuclear
7 structure is coupled to cytoskeletal organization and cellular morphology. Based on DAPI
8 images from our previous transduction experiments, we quantified changes in three-
9 dimensional nuclear shape, mechanical attributes and chromatin condensation state¹⁴. As
10 before, we observed that Lifeact expression altered nuclear state, giving raise to nuclei that
11 were up to 1.5 times larger in volume and less auxetic (**Fig. 4**), while chromatin
12 condensation remained unaffected (not shown). Again, we hypothesize that the effects of
13 Lifeact on the nucleus are a secondary result of alterations in cellular morphology and
14 cytoskeletal architecture¹⁴. Together, our results uncover for the first time that Lifeact-
15 induced artefacts on the actin cytoskeleton may have knock-on effects that extend into other
16 critical cellular structures.

17 18 **Lifeact-induced effects on the cytoskeleton lead to altered cell biophysical behaviour**

19
20 Having established the multiple effects of Lifeact on cellular structures, we moved to
21 examine their impact on cell biophysical behaviour. First, we used atomic force microscopy
22 to probe the nanomechanical properties of Lifeact-transduced cells. Our results showed a
23 mild decrease in cellular stiffness at very large peptide concentrations together with a steady
24 dose-response increase in cellular viscosity (**Fig. 5**). These results were initially surprising,
25 as we have previously shown a strong correlation between F-actin assembly and cellular
26 stiffness¹³. Nevertheless, it's worth stressing that cells with very large levels of Lifeact
27 expression displayed thick fibres disjointed from each other (cell #2 in **suppl. Fig 7**),
28 sometimes leaving between them large cell areas devoid of any actin-rich structure. This
29 scenario is thus very different from the previously described nematic phase of actin
30 organization¹⁵ (cell #1 in **suppl Fig 7**) and may rather resemble the liquid-like behaviour of
31 actin structures recently observed *in vitro* after coalescence and shortening of actin
32 bundles¹⁶.

33 Increasing evidence points towards the YAP/TAZ pathway as a crucial regulator of
34 cellular mechanosensing in stem cells¹⁷. In particular, the translocation of YAP into the cell
35 nucleus constitutes a hallmark of increased intra or extracellular forces that are transmitted
36 through the cytoskeleton and to the nucleus¹⁸. Accordingly, we set to quantify whether YAP
37 intracellular localization would be affected by Lifeact transduction, as a second evidence of

1 altered cell biophysical properties. To this end, we quantified the ratio of nuclear to cytosolic
2 YAP and observed that cells with higher Lifeact-GFP expression had lower amount of YAP
3 in the nucleus when compared to weakly-transduced cells (**Figure 6a**). Furthermore, we
4 explored whether the ratio of nuclear to cytosolic YAP correlated with cell spread area, as
5 found by others¹⁹. In control cells (not transduced) we found a constant value of nuclear to
6 cytosolic YAP ratio that was not modulated by cell area (**Figure 6b**). Conversely, for cells
7 transduced with Lifeact, nuclear to cytosolic YAP ratios were overall larger, and they tended
8 to decrease with increasing cell area (**Figure 6b**). This behaviour is reminiscent of that
9 observed in **Figure 5a** for cellular stiffness, and may reflect a mild decrease in intracellular
10 tension with increasing Lifeact expression that then results in decreased nuclear
11 translocation of YAP. Of note, immunostaining images of YAP used for this analysis showed
12 a striking unexpected feature, that is, Lifeact-dense stress fibres appeared to be decorated
13 with YAP, a feature that was not observed in control cells (**Figure 6c**). In both cases, the
14 preferred nuclear localization of YAP was preserved. Furthermore, we verified that this
15 observation was not due to bleed-through between the GFP and TRITC fluorescence
16 signals, or unspecificity of the TRITC-tagged secondary antibody used throughout this study
17 (**supplementary Figure 8**). Conversely, our analysis shows that YAP colocalization with F-
18 actin fibres increases with increasing Lifeact expression levels (**Figure 6d**).

19
20 As a third biophysical behaviour, we evaluated whether Lifeact expression would
21 affect cell motility by performing long-term live cell imaging 5 days post-transduction.
22 Individual cells were tracked by acquiring fluorescence images of the GFP channel every 10
23 minutes over a period of 18 hours and the resulting videos were later analysed using the
24 same image analysis pipeline as before. In addition to the parameters describing
25 cytoskeletal organization presented above, we also computed the total distance migrated by
26 each cell along with the directionality of migration (**Fig. 7**). We found that cells displaying low
27 Lifeact expression migrated for longer distances in a less directed fashion. Conversely, cells
28 with intermediate Lifeact expression tended to exhibit shorter but directionally-persistent
29 trails, consistent with our previous finding that these cells tend to display more aligned stress
30 fibers (**Supplementary Fig. 4 d, Supplementary videos**). Finally, cells with very high levels
31 of Lifeact expression exhibited severely impaired migration, remaining quasi-static and
32 erratic in their displacements. Of note, cells that had lower Lifeact expression did reorganize
33 their cytoskeleton to a larger extent in the timeframe of minutes, as shown by the frame-to-
34 frame changes in F-actin assembly (**Fig. 7 c**). Accordingly, we hypothesize that the impaired
35 migration displayed by cells expressing high levels of Lifeact expression is due to reduced F-
36 actin dynamics when reorganizing their cytoskeleton.

37

1 Impaired cofilin binding to F-actin as an underlying mechanism for the Lifact- 2 induced aberrations in actin organization and dynamics

3
4 Finally, we set out to pinpoint the potential mechanism by which Lifact alters F-actin
5 organization and dynamics. Cofilin was identified as a plausible key player, since Lifact has
6 been suggested by others to impair actin filament severing by cofilin both *in vitro* and in
7 yeast cells⁶. We thus carried out several experiments to assess if and how Lifact
8 expression led to reduced cofilin activity. On the one hand, we followed the procedure
9 devised by Hotulainen *et al.*, which elegantly show that the G-actin sequestering drug
10 Latrunculin A (LatA) fails to depolymerize the actin cytoskeleton when cofilin activity is
11 impaired²⁰. We incubated Lifact-transduced cells with LatA and simultaneously conducted
12 live-cell fluorescence imaging for 30 minutes at 2-minute intervals. By measuring the relative
13 drop in F-actin amount during treatment, we verified that Lifact reduced LatA-induced
14 cytoskeletal depolymerisation in an expression-dependent manner (**Fig. 8 a**). While this
15 experiment suggested that Lifact inhibits cofilin activity, it did not identify whether the
16 underlying mechanism is associated with chemical inactivation of cofilin (via phosphorylation
17 at serine residue 3²¹) or conformational changes of the f-actin filament upon Lifact binding
18 that prevent cofilin binding^{2,6}. Accordingly, we performed western blot measurements of
19 cofilin and p-cofilin expression levels for cell populations transduced with Lifact or controls
20 (**Fig. 8 b, Supplementary Fig. 9**). Cells transduced with Lifact displayed 81% increase in
21 overall cofilin expression, while the expression levels of p-cofilin increased only by 51%.
22 Together, these results suggest that Lifact-transduced cells have higher total amounts of
23 cofilin, and that a lower percentage of said cofilin is in the inactive phosphorylated state.
24 Finally, we performed immunostaining against cofilin to assess whether the drop in cofilin
25 activity was associated with changes in cofilin binding to F-actin. Following the approach
26 devised by Hayakawa *et al.*²² using fluorescence image quantification, we measured
27 fluorescence intensity levels of cofilin in pixels previously identified as corresponding to an F-
28 actin fibre, thus obtaining a measure of cofilin colocalization to F-actin. When we produced
29 dose-response curves, we found that cells with higher expression of Lifact had lower
30 amount of cofilin colocalization (**Fig. 8 c**). Collectively, our results reinforce the hypothesis
31 proposed by Courtemanche *et*, where Lifact binding to F-actin induces a conformational
32 change in actin filament structure which is then incompatible with subsequent cofilin
33 binding⁶. This hypothesis should be contextualized with recent findings on the dual activity of
34 cofilin, involving both severing and depolymerisation of actin filaments²³. Of note, saturation
35 of actin filaments with cofilin dramatically changes their dynamics towards a
36 depolymerisation-prone state from both barbed and pointed ends²³. Our findings, together
37 with those of others^{6,23}, support the hypothesis that prior binding of Lifact to actin filaments

1 would prevent cofilin saturation of said filaments, thus inhibiting cofilin-induced actin
2 depolymerisation and reducing overall actin filament dynamics.

3 4 **Discussion**

5
6 In summary, our results suggest that Lifeact-GFP induces dose-response alterations
7 in the actin cytoskeleton, likely stemming from altered cofilin activity and reduced filament
8 dynamics. The effects extend beyond the actin cytoskeleton, also affecting other cytoskeletal
9 structures and impairing the overall biophysical behaviour of cells.

10
11 Our findings are more strongly marked in undifferentiated human stem cells, which
12 may be due to a higher capacity to uptake the adenovirally-delivered Lifeact plasmid.
13 Nevertheless, we obtain similar dose-response trends in immortalized cell lines (NIH/3T3
14 and COS-7), thus expanding the range of cells types were Lifeact has been shown to induce
15 aberrant morphologies^{8,9,24}. It is worth stressing that, in our hands, Lifeact affects different
16 cells types to a different degree, and some cytoskeletal features more strongly than others.
17 An illustrative example of a trend being missing is the parameter stellate factor for COS-7
18 cells (supplementary figure 6b, blue symbols). Stellate factor measures the tendency of a
19 cell to display filopodia projections or protuberances (high value of stellate factor). Indeed,
20 COS-7 cells are rather smooth in their perimetral appearance, and don't typically extend
21 protrusions. Being that the case, it is difficult to see a trend towards decreasing this value,
22 because even in control conditions this value is low to begin with. Nevertheless, we note that
23 the dose-response trends are typically preserved between cell types, thus suggesting a
24 common origin of the observed changes. The Lifeact plasmid we used included a CMV
25 promoter, which has been shown by others to induce milder aberrations than pBABE and
26 CAG⁸. Of note, our results using a recombinant Lifeact-GFP protein show that the effect of
27 Lifeact is similar regardless of the way in which the DNA (or protein) is delivered and
28 expressed into the cell. Similarly, the GFP tag used (TagGFP2) is a next-generation
29 monomeric fluorescent protein, again being linked to milder aberrations than other
30 dimerization-prone GFP tags⁸. Our results are thus obtained in conditions identified by
31 others as conducive to fewest aberrant morphologies in terms of choice of promoter and
32 fluorescence protein tag used. In spite of that, we find a dose-response effect at all MOI and
33 conditions used, thus raising concerns on the use of Lifeact as a cytoskeletal marker.

34
35 Given that the effects of Lifeact in cytoskeletal organization exhibit a dose-response
36 behaviour with a saturation plateau, our results bring new light to the difficult compromise
37 during transduction optimization, that is, maximising the number of transduced cells while

1 reducing the number of cells which are either dead or with aberrant morphologies. Contrary
2 to expected, for all transduction protocols that we tested, the number of cells that are
3 transduced but not aberrant is constant and much lower than anticipated (<20%)
4 (**Supplementary Fig. 10**). Lifeact transduction protocols found in published literature vary to
5 a certain degree between laboratories and also according to cell lines used. It may then be
6 difficult to judge, when optimizing a transduction protocol, whether experiments are being
7 carried out in non-artifactual conditions based only on MOI estimates. We propose an
8 alternative approach, that is, that the presence of few clearly obvious aberrant cells (gray
9 bars in **Supplementary Fig. 10**) should be used as a tell-tale sign that a large percentage of
10 cells are within the dose-response regime (white bars in **Supplementary Fig. 10**) and that
11 few cells will be truly non-artifactual. We note here that transduced cells that display minor
12 aberrations are likely to go unnoticed to the naked eye during the course of an experiment.
13 Selection of these cells in a study will lead to experimental bias or lack of reproducibility with
14 results obtained using other live cell actin probes. Prior to performing experiments, it is
15 important researchers establish a reliable protocol to identify and select only suitable cells
16 within the whole population of heterogeneously transduced cells. Similarly, it would be
17 advisable to report the percentages of not-affected, aberrant and grossly-aberrant cells
18 within the cell population for any given transduction protocol used in a study. Preliminary
19 tests based on co-staining with an actin marker such as phalloidin and image quantification
20 at the single cell level can provide this type of information in a swift manner. With this study,
21 we hope to start an active discussion on what are the limits of suitability of our current live-
22 cell cytoskeletal reporters. This is a timely and much-needed debate, especially with the
23 advent of other actin reporters, such as SiR-actin, Utrophin or F-tractin, which may display
24 similar associated issues.

25
26

27 **Methods**

28 **Cell culture, Lifeact-GFP transduction and immunostaining.** Unless stated otherwise, all
29 chemicals and reagents were obtained from Sigma. The majority of measurements were
30 performed in human bone marrow derived mesenchymal stem cells (Promocell), while
31 additional measurements were performed in NIH/3T3 and COS-7 cells. Cells were
32 maintained in culture medium consisting of low glucose Dulbecco's Modified Eagle Media
33 (Gibco) supplemented with 10% foetal bovine serum, and 100U/ml Penicillin- 100µg/ml
34 Streptomycin. hMSCS were additionally supplemented with 10 ng/ml fibroblast growth factor
35 (Peprotech). Cells were kept in tissue culture flasks and cultured at 37°C and 5% CO₂.
36 Mesenchymal stem cells were used between passages 5 and 9. Lifeact-GFP transductions

1 were performed using commercial rAV-CMV-LifeAct-TagGFP2 Adenoviral Vectors (Ibidi)
2 according to supplier's instructions, by addition of viral transduction reagent volumes
3 required to achieve the desired MOI (i.e. 100, 300, 600 or 1000) on each sample. After the
4 initial 18 hours of incubation for vector uptake, media containing viral particles was
5 exchanged. Cell samples were allowed to express Lifeact-GFP for a total of 1, 3, 5 or 7 days
6 prior to fixation. The pCMV-EGFP plasmid was a kind gift from Dr Julien Gautrot. For
7 experiments using recombinant Lifeact-GFP protein, Lifeact-TagGFP2 peptides and
8 proprietary Fuse-it-P intracellular protein delivery kits were acquired from Ibidi and prepared
9 according to instructions. Briefly, hMSCs were seeded into coverslips inside 6-well TCP
10 vessels, three days before experiments. Lyophilised peptides were reconstituted in sterile
11 water, and further diluted in 20mM HEPES buffer to a concentration of 0.1 mg/ml. Fuse-it-P
12 was loaded with peptides by following supplier's instructions. Cells were washed in PBS, and
13 1ml of 1:50 fusogenic mixture was dispensed to each well. After incubation for 5 minutes at
14 37°C, fusogenic mixture was replaced with cell culture medium and returned to an incubator.
15 Cell samples were fixed after 6 hours, to mitigate toxicity effects, stained and imaged as
16 before. All live cell experiments (migration, AFM and Latrunculin-A treatment) were
17 conducted on cells transduced at MOI 1000, at 5 days post transduction. The same
18 conditions were used for NIH3/3 and COS-7 cells. At least 3 independent transductions were
19 performed for each set of experiments.

20 For live cell imaging experiments, cells were directly plated onto 6-well plates and
21 cultured in FBS and antibiotic supplemented Flurobrite-DMEM imaging specific media
22 (Thermofisher). For AFM measurements, cells were plated in petri dishes and imaging
23 media were supplemented with 50 mM HEPES. For immunostaining experiments, cells were
24 sparsely seeded onto serum coated coverslips inside sterile petri dishes at least 1 day prior
25 to transductions. In brief, cells were fixed by treatment with 3.7% paraformaldehyde in PBS
26 for 15 min and permeabilised for 5 min in 0.25% Triton X-100. To visualize simultaneously F-
27 actin via Lifeact and Phalloidin, cells were stained with phalloidin-TRITC at 2 µg/ml in PBS
28 for 2 hours. For additional immunostaining experiments to visualize other cytoskeletons and
29 proteins, permeabilized cells were treated overnight with primary antibodies against vimentin
30 (1:400 dilution; RV202), α-tubulin (1:50 dilution; TU-02), YAP (1:200, 63.7) and cofilin (1:200;
31 E-8) diluted in goat serum blocking buffer at 4 °C (all antibodies mouse monoclonal from
32 Santa Cruz Biotechnologies). The next morning, the samples were washed with PBS and
33 treated with a TRITC-tagged secondary antibody (1:400 dilution, goat anti-mouse IgG-
34 TRITC, sc-3796) for 1 hour at room temperature. All coverslips were mounted onto glass
35 slides using ProLong® Gold Antifade Mountant containing DAPI (Thermo Fisher). Control
36 samples were cultured and stained in parallel to transduced cell cultures, but without having
37 been subjected to the transduction protocol.

1

2 **Quantification of cell morphology, cytoskeletal structures and nuclear state from**
3 **fluorescence images.** All fixed samples were imaged using an inverted epifluorescence
4 microscope (Leica DMI4000B) with a x20/0.50 NA objective lens and a CCD camera (Leica
5 DFC300FX). Cells were sequentially imaged on the DAPI (nuclei), TRITC
6 (phalloidin/antibody staining), and FITC (Lifeact-GFP) channels. The algorithm for single-cell
7 quantification of cytoskeleton structures has been described in previous publications^{13,14}.
8 The coded algorithm (CSKMorphometrics) has been implemented in MATLAB (Mathworks)
9 and can be found at the File Exchange repository at MATLAB central site. In brief, the
10 quantification of cell morphology and cytoskeleton configuration is based on three steps: (1)
11 initial fibre segmentation, (2) fibre refinement, and (3) determination and subtraction of
12 background. These steps output a variety of maps representing either the brightness of
13 segmented fibres or local fibre orientation, that allow subsequent estimation of morphometric
14 parameters for individual cells. This information is assembled into 14 descriptors
15 (Supplementary Information), e.g. cell spread area, total fibre amount. For the present study,
16 we use the term 'fibre amount' to signify the amount of protein organized in fibres, that is,
17 identified by the pipeline as part of the segmented cytoskeleton in the raw image.

18 Quantification of nuclear features to estimate relative nuclear mechanical parameters
19 is described elsewhere¹⁴. With this method we process DAPI-stained nuclei images to
20 quantify nuclear volume, Poisson ratio and chromatin state in individual cells.

21 Finally, the total intensity from GFP images belonging to individual cells was used as
22 a metric for intracellular Lifeact amount to produce graphs correlating cellular morphometrics
23 with peptide expression. Total GFP intensity was measured by adding up the fluorescence
24 intensity measured for all pixels within the outline of a cell, once background intensity was
25 subtracted. To statistically identify the three regimes in the dose-response curves, namely a
26 no effect regime, a dose-response regime, and a saturation plateau, threshold points were
27 calculated across all parameters by adapting a method previously developed by us and
28 based on the ratio of variances (RoV)²⁵ around each point of a DRC (**Fig. 2-8 and**
29 **Supplementary Fig. 4**). Briefly, a test parameter RoV is defined as $RoVi = \frac{var(di+1:di+N)}{var(di-N:di-1)}$, i.e.
30 the ratio of the variances computed in two N-sized small windows to each side of every point
31 i in each DRC. Peaks in RoV displaying regions of high variability in the data, signifying a
32 transition between regimes, were identified in each DRC curve. Two global GFP intensity
33 values corresponding to the transitions point to dose-response and saturation regimes were
34 obtained by averaging out all threshold GFP intensities obtained in **figures 2-8 and**
35 **supplementary figure 4**. The values for the two global GFP intensity thresholds are

1 included in **supplementary fig 2** and were used to sort individual cells into the 3 regimes
2 depicted in **supplementary figure 10**.

3

4 **Quantification of Nuclear/Cytosolic ratio of YAP** . Nuclear/Cytosolic ratio of YAP was
5 assessed as previously described by others¹⁸. Briefly, we measured the average
6 fluorescence intensities of YAP staining in the nucleus and in an annular region with equal
7 size in the cytosol immediately adjacent to the nuclear region, and computed their ratio.

8

9 **Western Blotting.** Cells were washed with chilled PBS and lysed in RIPA buffer for 15 min
10 on ice. The total protein concentration was determined by the BCA assay. Cell lysates were
11 mixed with Laemmli buffer and denatured by heating at 100°C for 5 min. Proteins were
12 separated by SDS–PAGE and transferred onto a nitrocellulose membrane. Membranes were
13 blocked in 5% dry milk for 1 h, followed by incubation with primary antibodies for cofilin
14 (1:125, E-8, Santa Cruz), p-cofilin (1:250, E-5 Santa Cruz) and control glyceraldehyde 3-
15 phosphate dehydrogenase (GAPDH) (1:500, 0411, Santa Cruz) over night at 4 °C. Excess of
16 antibody was removed by washing with PBST three times and the secondary antibody
17 donkey anti-mouse (IRDye® 680RD Donkey anti-Mouse IgG (H + L), [P/N 926-68072];
18 1:10000) was added for 1h at room temperature in dark. The proteins recognized by the
19 antibody were visualized by chemiluminescence. ImageJ was used to quantify the intensity
20 of cofilin, p-cofilin and GAPDH protein bands from each blot.

21

22 **Migration and cytoskeleton disassembly experiments.** For migration and Latrunculin-A
23 treatment experiments, live-cell imaging was performed under temperature and CO₂
24 controlled environment, using an incubator-encased epifluorescence imaging system
25 (Lumascop 720, Etaluma) at x20 magnification. Transduced cells were cultured inside 6-
26 well plates until the time of imaging. Individual cells were continuously tracked for 18 hours
27 at 10-minute intervals, and imaged in the FITC channel. To produce Supplementary videos
28 of long term behaviour in Lifact-GFP expressing cells, imaging was conducted under similar
29 conditions using 10x magnification for a period of 4 days, sampled at 1-hour intervals.
30 Control cells remained untransduced for the duration of the experiment. Other conditions
31 consisted of cells transduced at MOIs of 250 or 500.

32 To characterize migration patterns, every frame on the 18 hours time-lapse video
33 pertaining to the Lifact-GFP channel was analysed using the formerly described image
34 processing algorithms. The positions of cell centroids were tracked from masks of
35 instantaneous cell shape and used to quantify total distance migrated. Migration

1 directionality was defined as the ratio between net cell displacement (the euclidian distance
2 between starting and ending centroid positions) and the overall distance travelled by the cell,
3 as $MD = \frac{d(P_{t=0}, P_{t=T})}{\sum_{i=0}^T d(P_{t=i}, P_{t=i+1})}$. F-actin interframe change was calculated comparing values of F-
4 actin (FA) between successive frames, as $IFC_i = 100 \cdot \frac{FA_{i+1} - FA_i}{FA_i}$. For cytoskeleton
5 disassembly studies, cells were imaged for 30 minutes at 2-minute intervals immediately
6 upon addition of Latrunculin A (0.075µg/ml) to the culture medium. F-actin disassembly was
7 quantified as $100 \cdot \frac{FA_{t=0min} - FA_{t=30min}}{FA_{t=0min}}$

8
9
10 **Determination of cellular stiffness and viscosity with atomic force microscopy.** All
11 measurements of cell mechanics were performed on a Nanowizard 4 (JPK), integrated with
12 an Axio Observer Z.1 epifluorescence microscope with Plan-Apochromat lenses (20x)
13 equipped with a cooled CMOS camera (Orca Flash 4). Cells were probed using gold-coated
14 rectangular cantilevers (0.03 N/m nominal spring constant) with pyramidal tips (12 µm high
15 with 35° half cone angle, supplied by BudgetSensors). Experiments were conducted on petri
16 dishes mounted on a heating accessory to maintain cells at 37° C. AFM experiments were
17 conducted for a maximum of 1hr per petri dish. Prior to measurement, the cantilevers were
18 allowed to thermally equilibrate fully submerged in cell media. The cantilever sensitivity was
19 calibrated in contact mode on a bare region of the container, following which the cantilever
20 was moved a minimum of 500 µm from the surface to calibrate the force constant using
21 thermal fluctuations. We identified individual adherent cells exhibiting varied levels of GFP
22 expression and recorded a fluorescence image of the GFP channel at 20× magnification
23 before measuring cell mechanics. Imaging parameters (exposure time and gain) were kept
24 constant for all experiments. AFM measurements were performed using JPK's QI mode,
25 which rapidly acquires force-curves generating a detailed image of the topography and
26 mechanical properties of the sample. For each measurement we selected a region of 100 by
27 100 µm (32x32 force curves) ranging from lamellar and cytosolic to nuclear regions of the
28 cell. Force curves had a z-length of ~10 µm, extension speed of 125 µm/s and a setpoint of
29 3-5 nN.

30 Data analysis of the force-displacement curves was carried out using the BECC
31 model for thin adherent cells on a stiff substrate²⁵ using a pipeline written in MATLAB as
32 previously described²⁵. Cellular viscosity was computed using the same force-displacement
33 curves following the method outlined by Rebelo *et al*²⁷.

1 **Statistical Analysis.** Statistical tests were produced with the OriginLab analysis software.
2 Population results were plotted as box charts presenting median values and first and third
3 quartiles, with error bars indicating the 1st and 99th quartiles. Single cell results were
4 expressed either as means or geometric means with error bars representing interquartile
5 range. Two-way ANOVA tests were used to establish the significance of concentration and
6 time effects on the levels of Lifeact expression and of morphological alterations of cell
7 populations. Dunnett's post-hoc tests were used to determine significant differences
8 between the control group (no transduction) and groups treated with increasing MOIs for
9 each day measured.

10

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32 atomic force microscopy. *Nanotechnology* **24**, 055102 (2013).
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35

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8

9 **Author contributions**

10 L.R.F. and N.G designed the studies; L.R.F., M.C.K., X.Z. and K.S. conducted experiments.
11 All authors contributed to analysing and interpreting the data. L.R.F. and N.G drafted the
12 manuscript and all authors edited the final submission.

13

14 **Competing interests**

15 No competing interests declared.

16

1 **Figure captions**

2 **Figure 1** | Characteristic phenotypes of cells expressing increasing amounts of Lifeact-GFP
3 and co-stained with phalloidin-TRITC and DAPI. (a) Cell cultured on a coverslip dish that
4 was not transduced, (b) cell sorted as 'no-effect regime', (c) cell sorted as 'dose-response-
5 regime', (d) cell sorted as 'saturation plateau'. Scale bar corresponds to 15 μm and is the
6 same for all cells pictured.

7

8 **Figure 2** | Dose-response curves quantifying the effect of Lifeact expression in cell spread
9 area (a), cell perimeter stellate factor (b), aspect ratio (c), f-actin amount (d), fibre thickness
10 (d) and chirality of fibres (f). Values for >100 cells were pooled together to compute each
11 individual data point. Data is presented as geometric mean (a and d), mean (b and e) or
12 median (c and f) error bars indicate geometric standard deviation, standard deviation or Q1-
13 Q3, accordingly. Background colours indicate the regimes where cells display no Lifeact-
14 induced effect (yellow background), a dose-response trend (white background) and a
15 saturation plateau (gray background), as identified from analyses of peak changes in
16 variability in the neighbourhood of each point for each parameter plotted.

17

18 **Figure 3** | Lifeact-driven effects extend to non-actin-based cytoskeletal networks.
19 Quantification of Lifeact effects on intermediate filaments assembly (a) and microtubule
20 assembly (b). Values for >40 cells were pooled together to compute each individual data
21 point. Data is presented as geometric mean, error bars indicate geometric standard
22 deviation. Background colours indicate the regimes where cells display no Lifeact-induced
23 effect (yellow background), a dose-response trend (white background) and a saturation
24 plateau (gray background), as identified from analyses of peak changes in variability in the
25 neighbourhood of each point for each parameter plotted.

26

27 **Figure 4** | Lifeact-driven effects modulate nuclear state. Quantification of Lifeact effects on
28 nuclear volume (a) nuclear Poisson's Ratio (b). Values for >40 cells were pooled together to
29 compute each individual data point. Data is presented as mean, error bars indicate standard
30 deviation. Background colours indicate the regimes where cells display no Lifeact-induced
31 effect (yellow background), a dose-response trend (white background) and a saturation
32 plateau (gray background), as identified from analyses of peak changes in variability in the
33 neighbourhood of each point for each parameter plotted.

1 **Figure 5** | Lifact expression alters cellular mechanical properties. Lifact dose dependent
2 effects on cell stiffness (a), and viscosity (b) Values for >10 cells were pooled to compute
3 each individual data point. Data is presented as geometric mean, error bars indicate
4 geometric standard deviation. Background colours indicate the regimes where cells display
5 no Lifact-induced effect (yellow background), a dose-response trend (white background)
6 and a saturation plateau (gray background), as identified from analyses of peak changes in
7 variability in the neighbourhood of each point for each parameter plotted.

8

9 **Figure 6** | Lifact expression alters intracellular localization of YAP. Ratio of nuclear to
10 cytoplasmic YAP localization according to Lifact-GFP cellular fluorescence (a) and cell area
11 (b). In (b), black symbols correspond to cells not transduced (control) and red symbols
12 correspond to cells transduced with Lifact-GFP. (c) Example cells displaying localization of
13 YAP staining to Lifact-containing stress fibres. The cell on the left was transduced with
14 Lifact and the cell on the right was not transduced. After fixation, cells were stained with
15 DAPI (middle panels) and against YAP (bottom panels). Scale bar is 50 μm . (d) Average
16 pixel intensity of YAP fluorescence colocalized to Lifact-containing stress fibres is
17 dependant on the total amount of Lifact expressed in the cell. Data is presented as mean,
18 error bars indicate standard deviation. For (a) and (d), background colours indicate the
19 regimes where cells display no Lifact-induced effect (yellow background), a dose-response
20 trend (white background) and a saturation plateau (gray background), as identified from
21 analyses of peak changes in variability in the neighbourhood of each point for each
22 parameter plotted. Values for >12 cells were pooled to compute each individual data point.

23

24 **Figure 7** | Lifact expression alters cell migration and F-actin dynamics. Lifact dose
25 dependent effects on distance migrated (a), directionality of migration (b) and F-actin inter-
26 frame changes (c). Values for >5 cells were pooled to compute each individual data point.
27 Data is presented as geometric mean, error bars indicate geometric standard deviation.
28 Background colours indicate the regimes where cells display no Lifact-induced effect
29 (yellow background), a dose-response trend (white background) and a saturation plateau
30 (gray background), as identified from analyses of peak changes in variability in the
31 neighbourhood of each point for each parameter plotted.

32

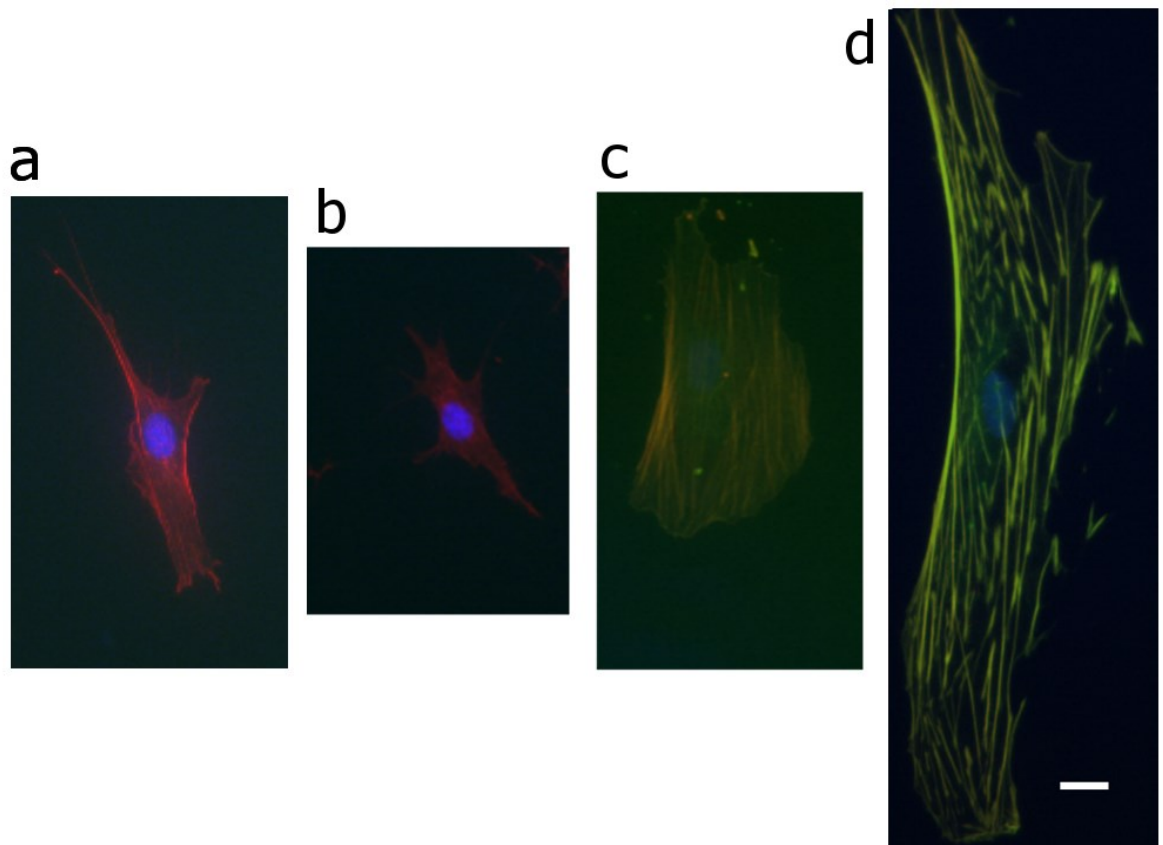
33 **Figure 8** | Lifact expression alters cofilin activity. (a) Lifact dose dependent effects on F-
34 actin disassembly after 30 min of Latrunculin A (0.075 $\mu\text{g/ml}$) treatment. (b) Western blot

1 results for cofilin and p-cofilin expression levels relative to GADPH. (c) Lifeact dose
2 dependent effects on fluorescence intensities of cofilin colocalized to F-actin fibres. For (a)
3 and (c), data is presented as median, error bars indicate Q1-Q3, N>100 cells; for (b) data is
4 presented as mean, error bars indicate standard deviation, N=3 repeats.

5

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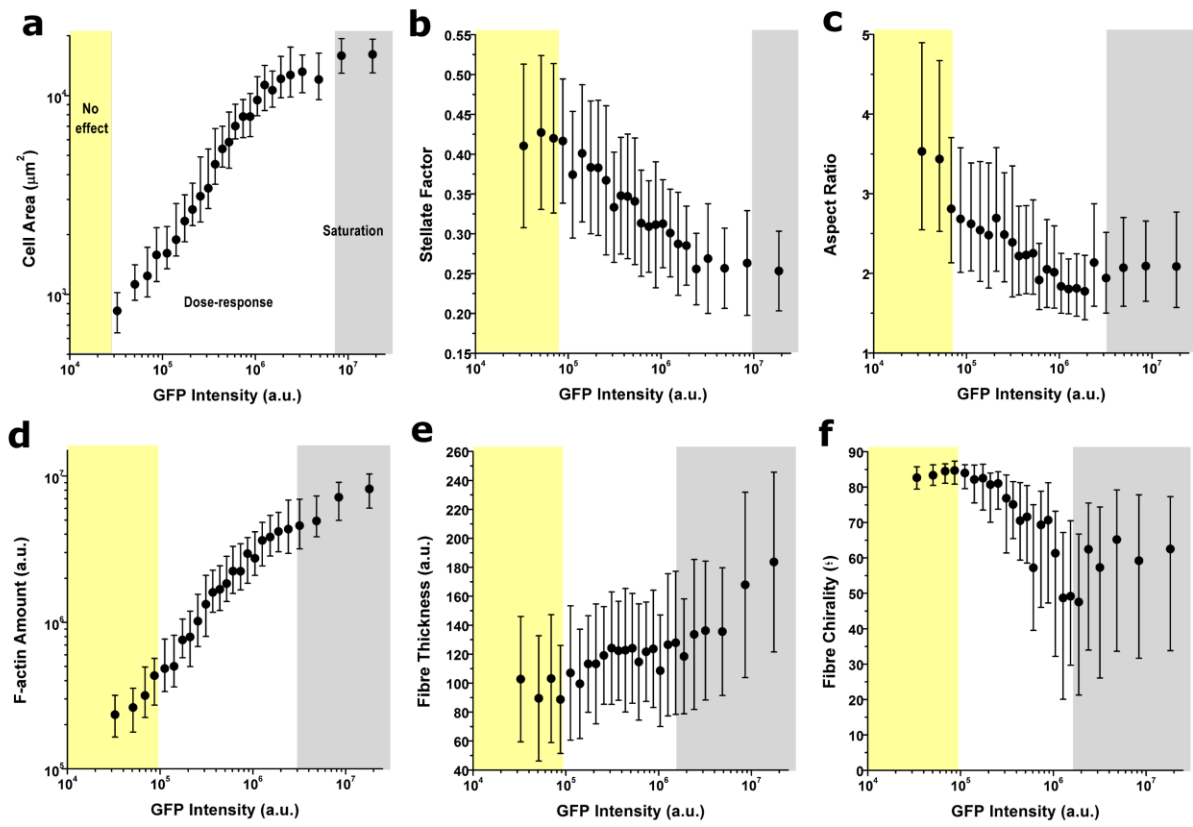
1 Figure 1



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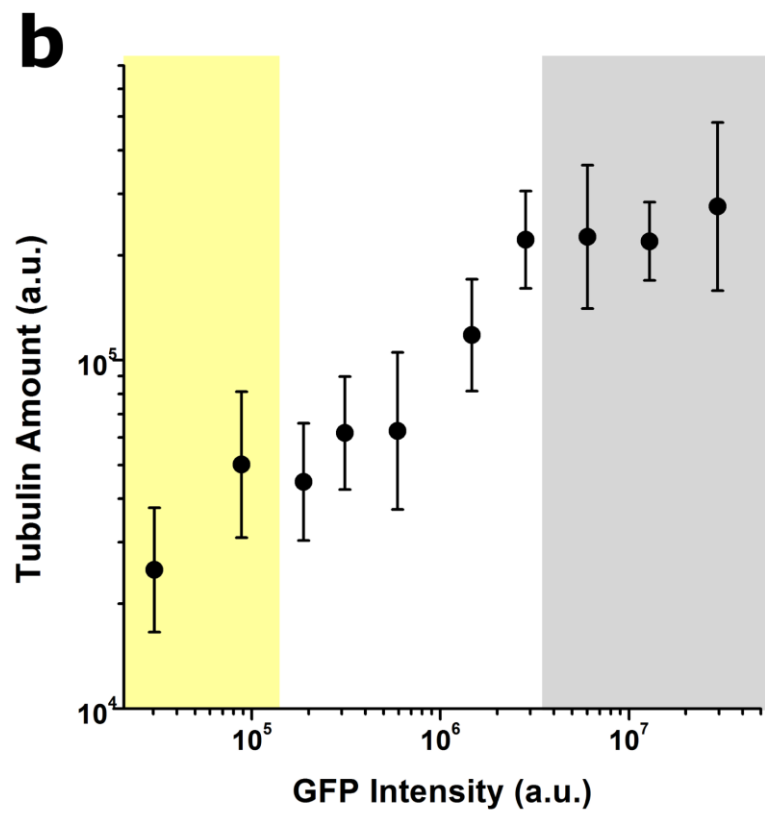
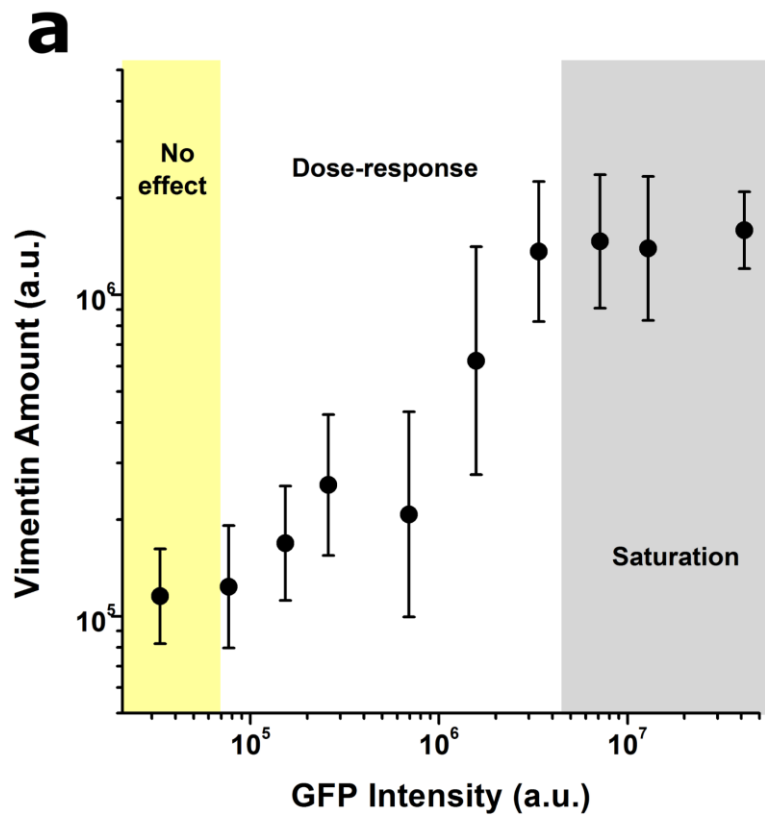
1 Figure 2



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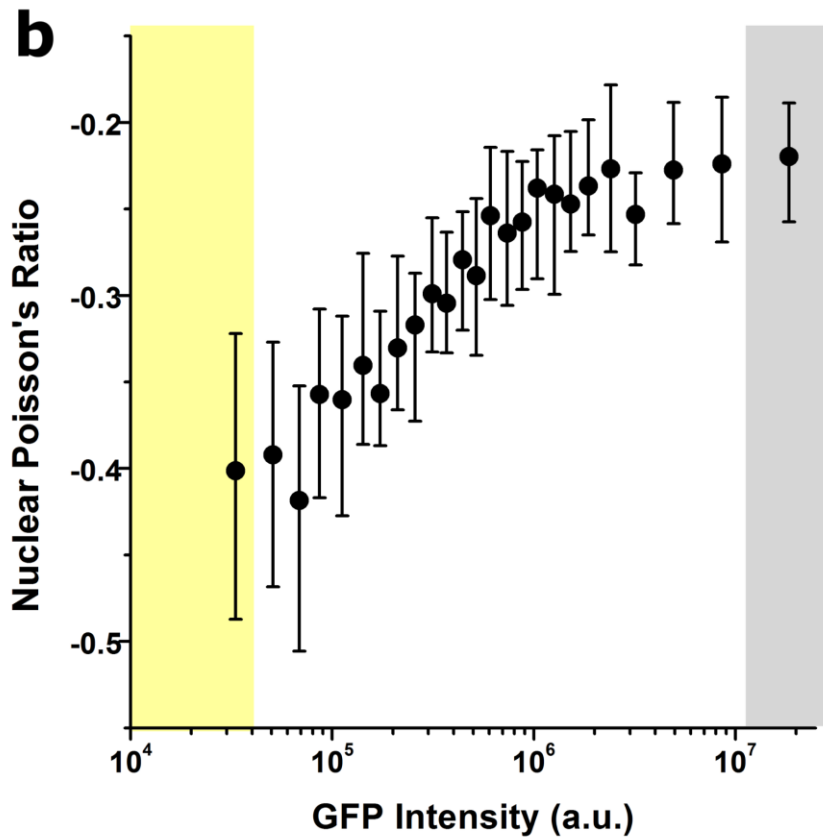
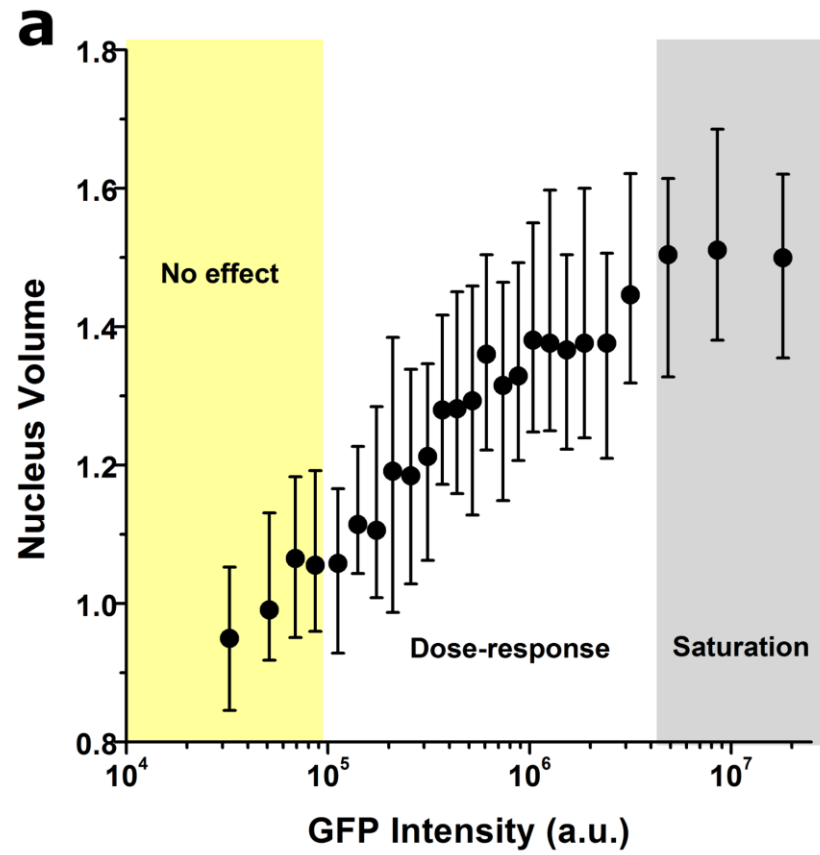
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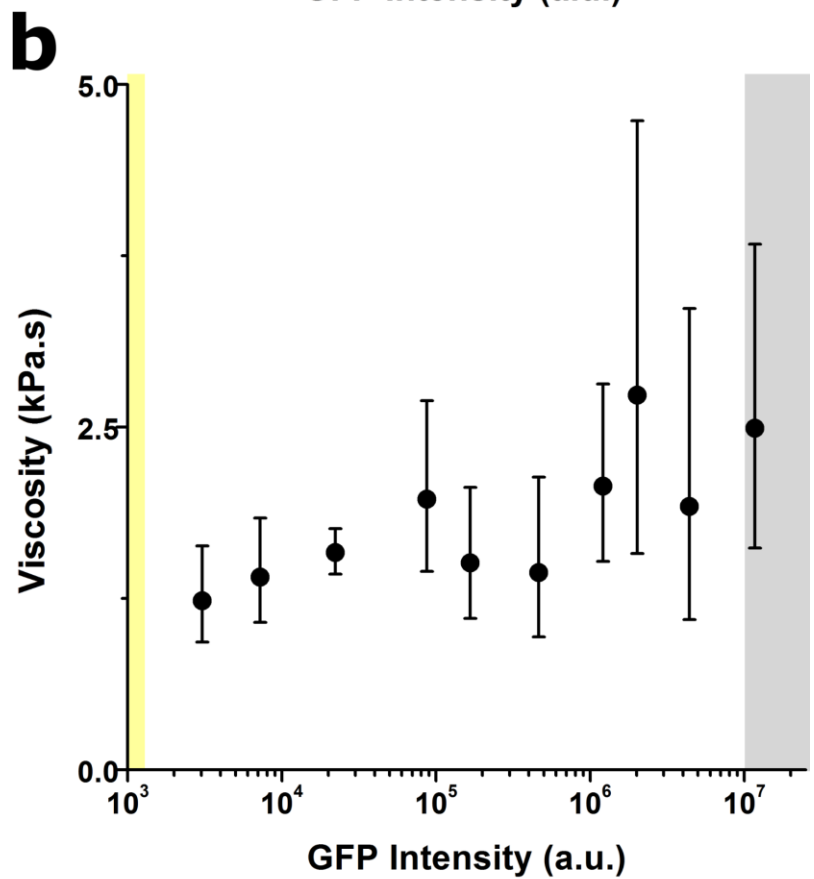
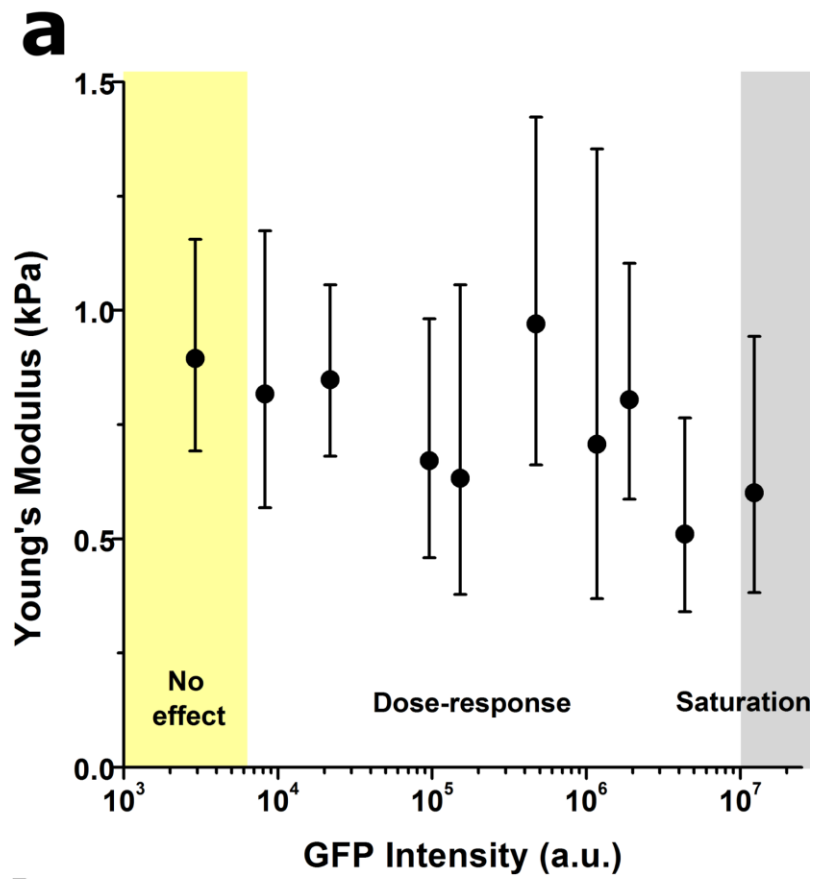
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1 Figure 4



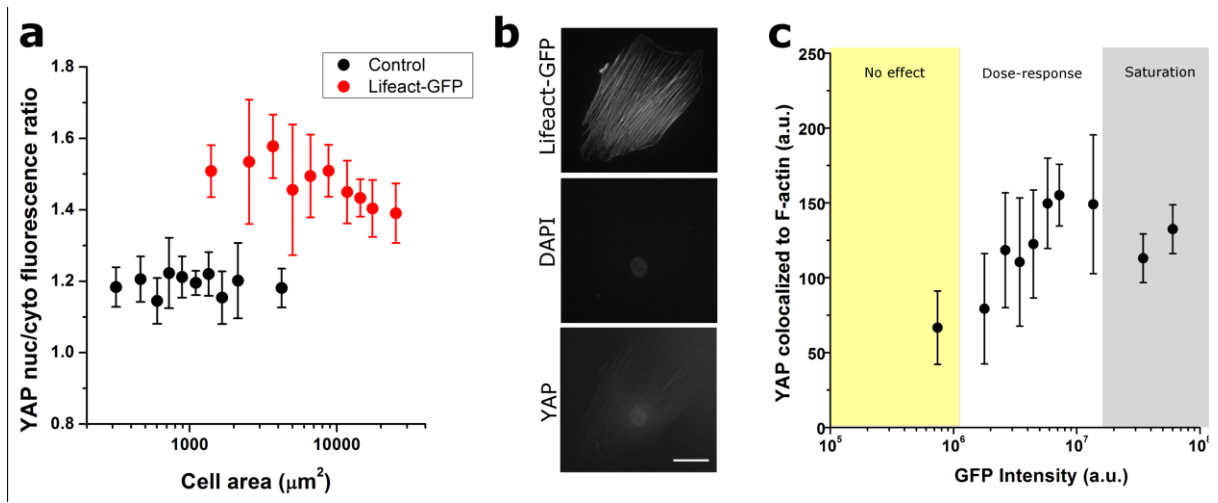
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1 Figure 5



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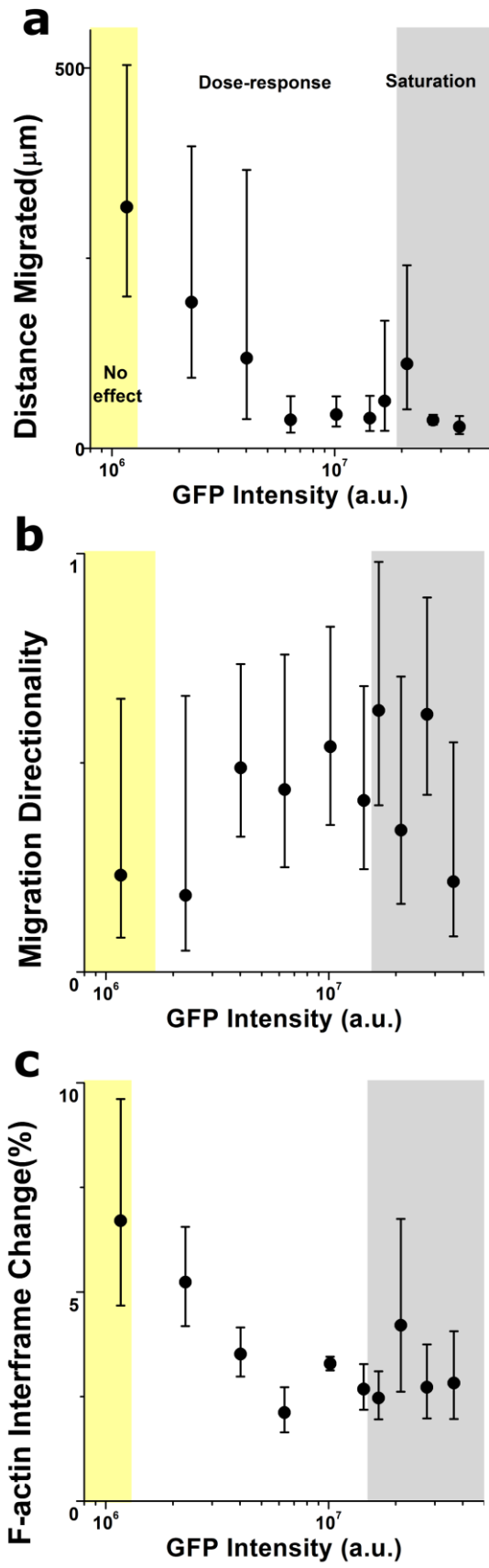
1 Figure 6



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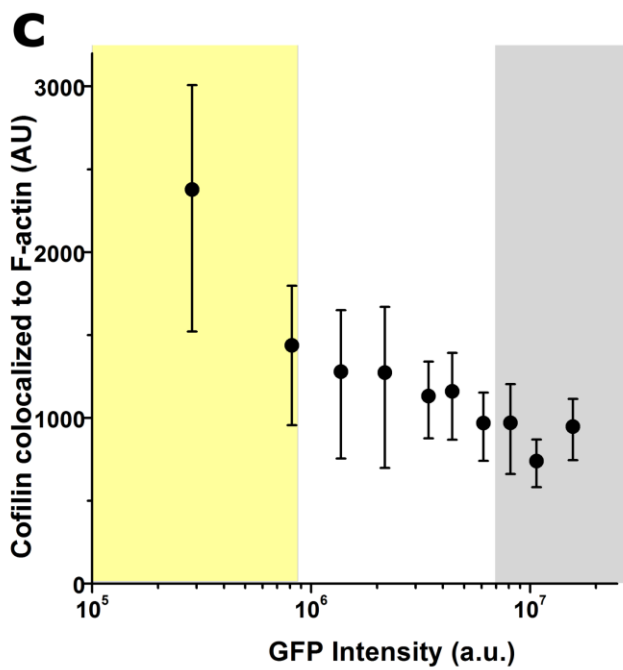
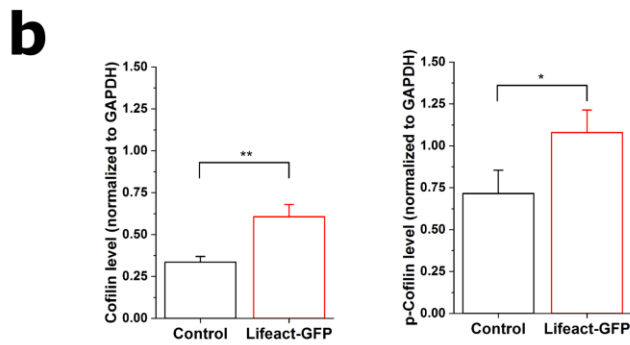
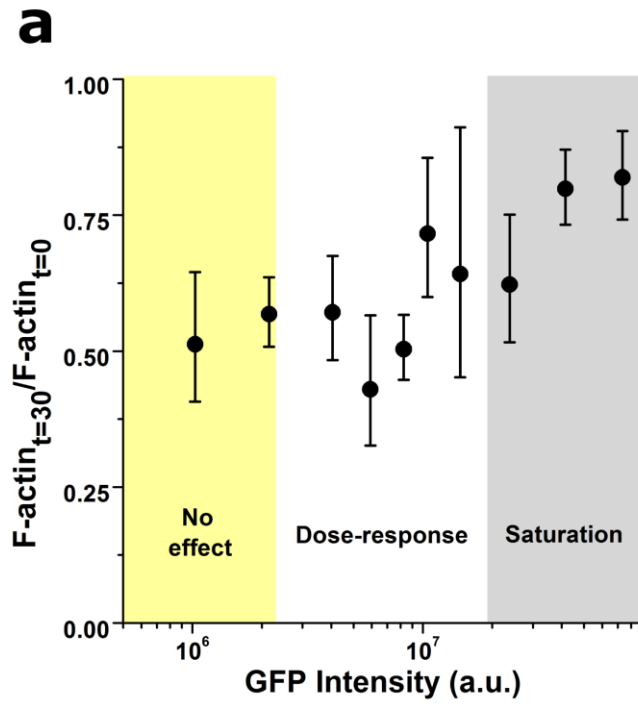
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1 Figure 7



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1 Figure 8



2