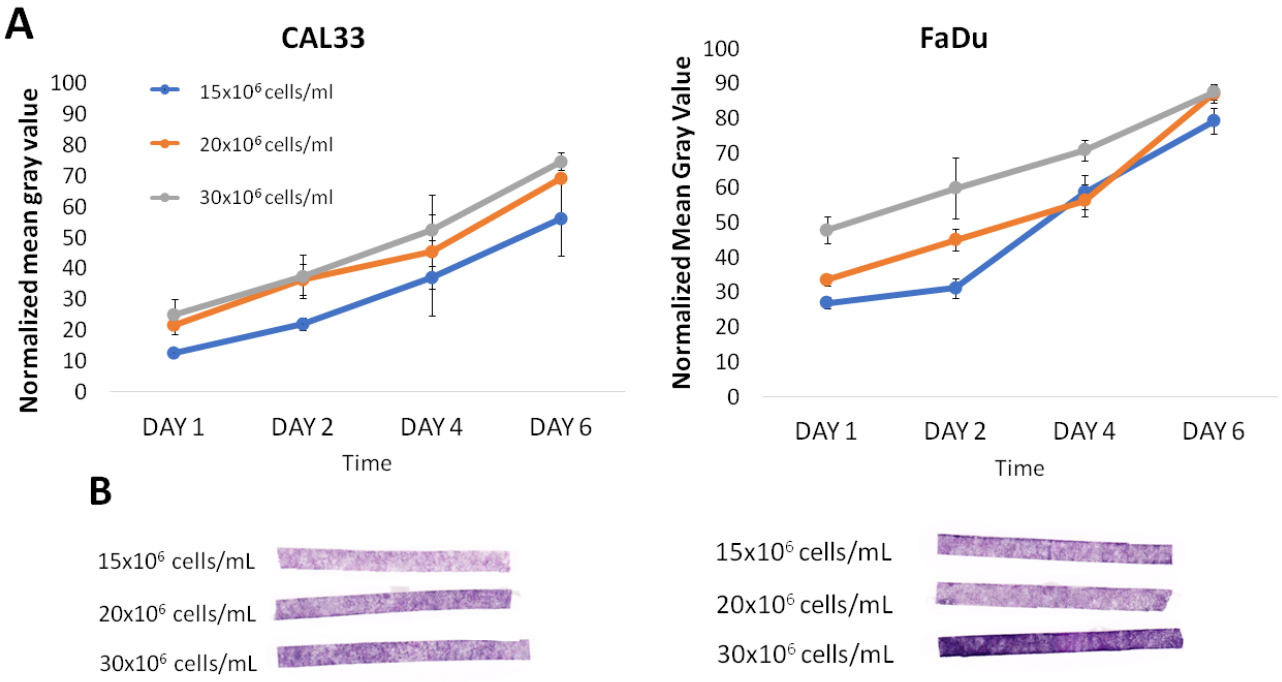
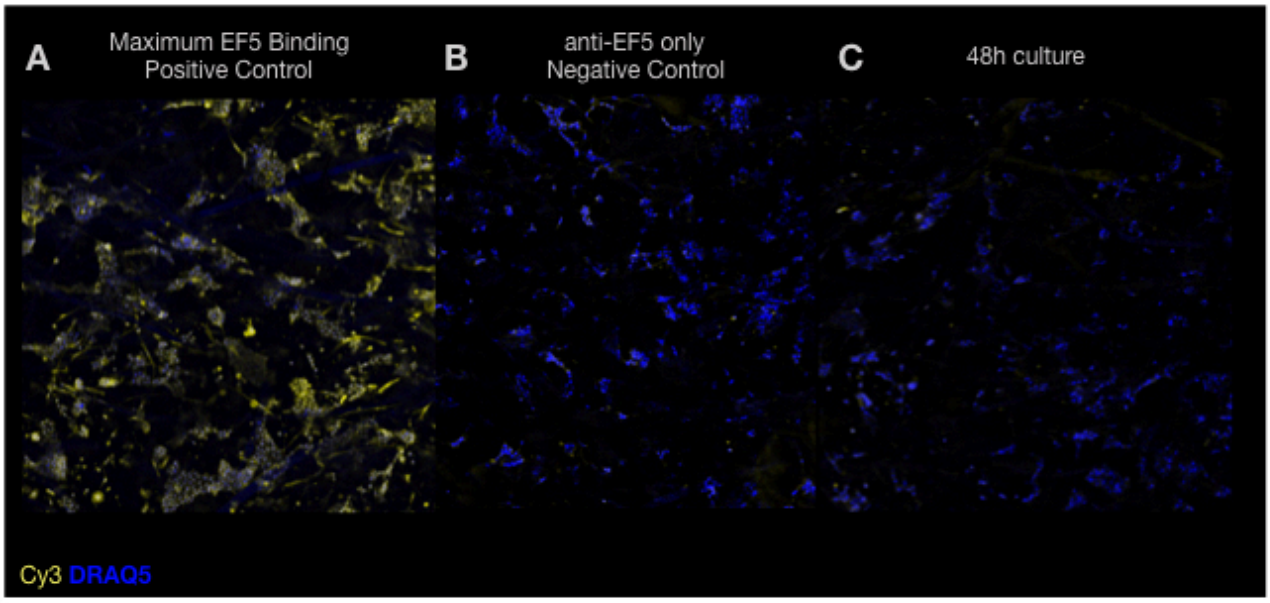
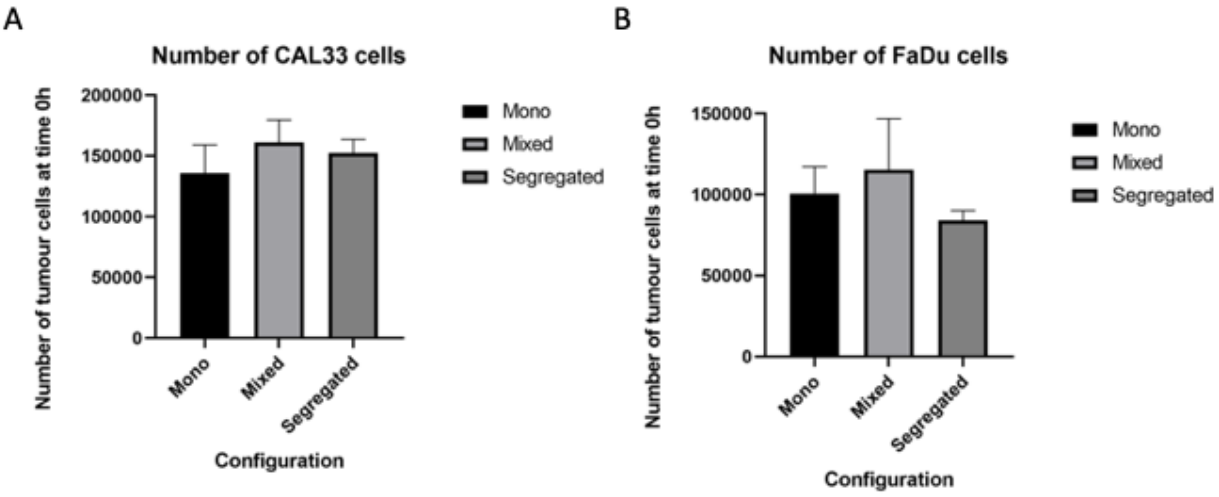
Dean et al. Supplementary Information



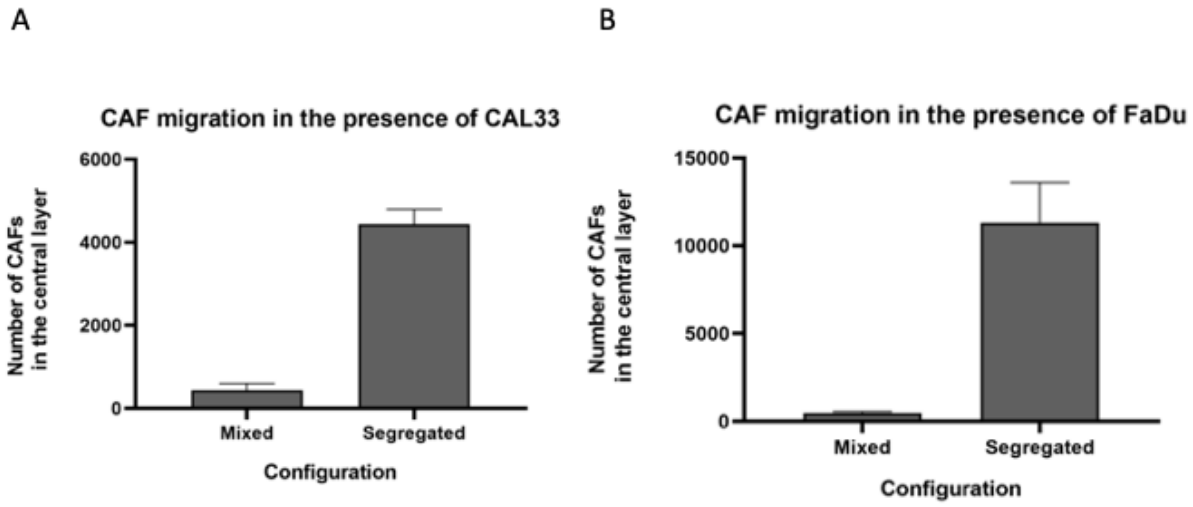
SI Figure 1 Optimization of biocomposite cell seeding density. A), CAL33 cells (left) or FaDu cells (right) did not reach a growth plateau within a 6-day period for any cell seeding density tested. Biocomposites (never rolled) were treated with 0.05 mg/mL of MTT in cell culture media for 2 hours following 1, 2, 4, or 6 days seeding at seeding densities of 15, 20, or $30x10^6$ cells/mL. Values on graph represent mean ± SEM of 3 independent experiments. B), Images of CAL33 (left) and FaDu (right) biocomposites following treatment with MTT, 2 days post seeding.



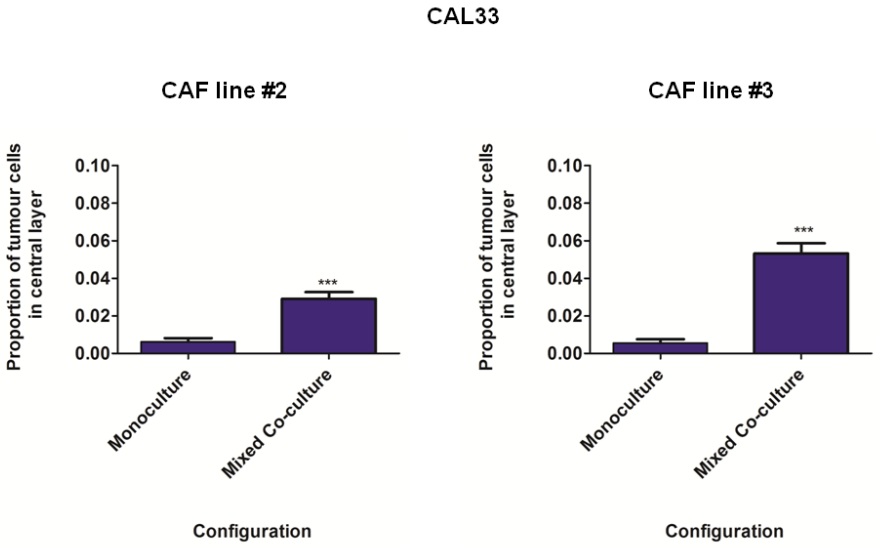
SI Figure 2: Assessment of Hypoxia in Mixed Coculture Configuration
Mixed co-culture constructs were rolled for 48h and assessed for hypoxia using EF5 staining. Representative images showing EF5 labelled with Cy3 (yellow) and nuclei labelled with DRAQ5 (blue) for samples stained in (A) anoxia, stained with (B) anti-EF5 only, and after (C) 48h rolled culture.



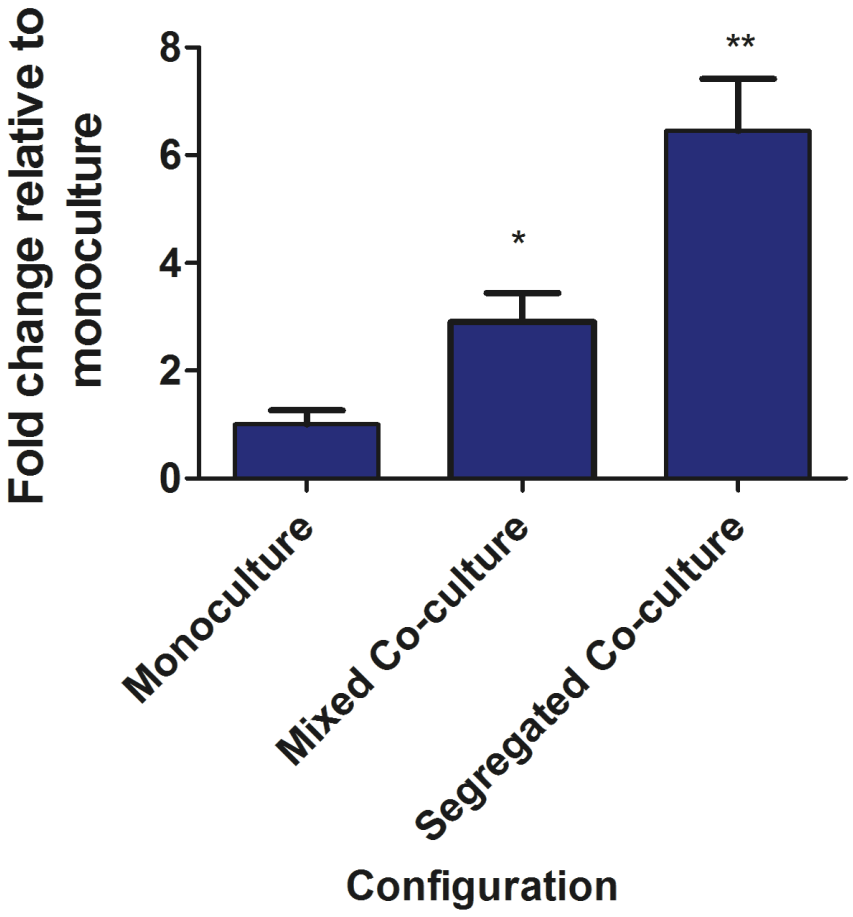
SI Figure 3: Number of tumour cells present in the seeded biocomposite at 24h after fabrication in monoculture versus co-culture. There were no significant differences in the number of tumour cells after a 24h pre-culture prior to rolling for CAL33 (A) and FaDu (B) TRACERs.



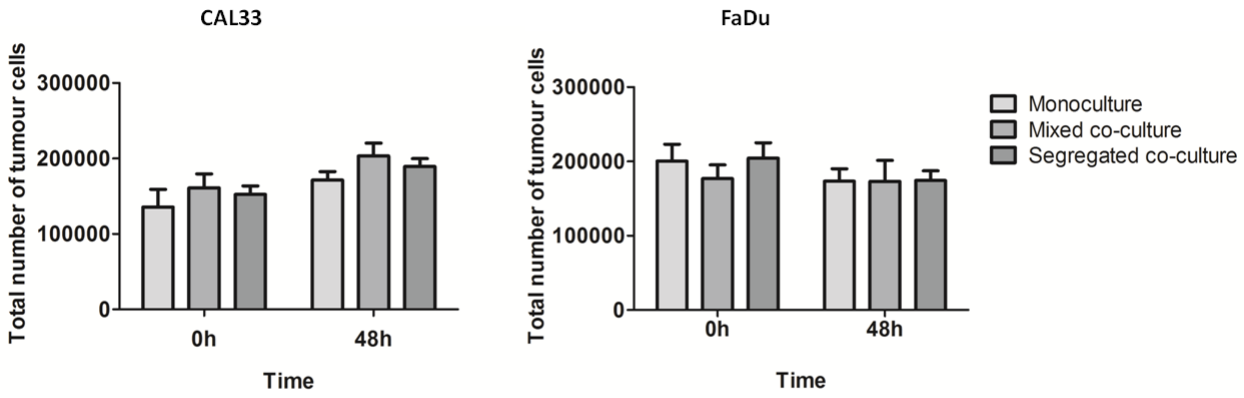
SI Figure 4: CAF invasion in mixed and segregated TRACER co-culture.
CAF migration toward the central layer of TRACER in response to CAL33 (A) or FaDu cells (B) after 48 h of rolled culture. Bars represent mean ±SEM for three independent experiments.



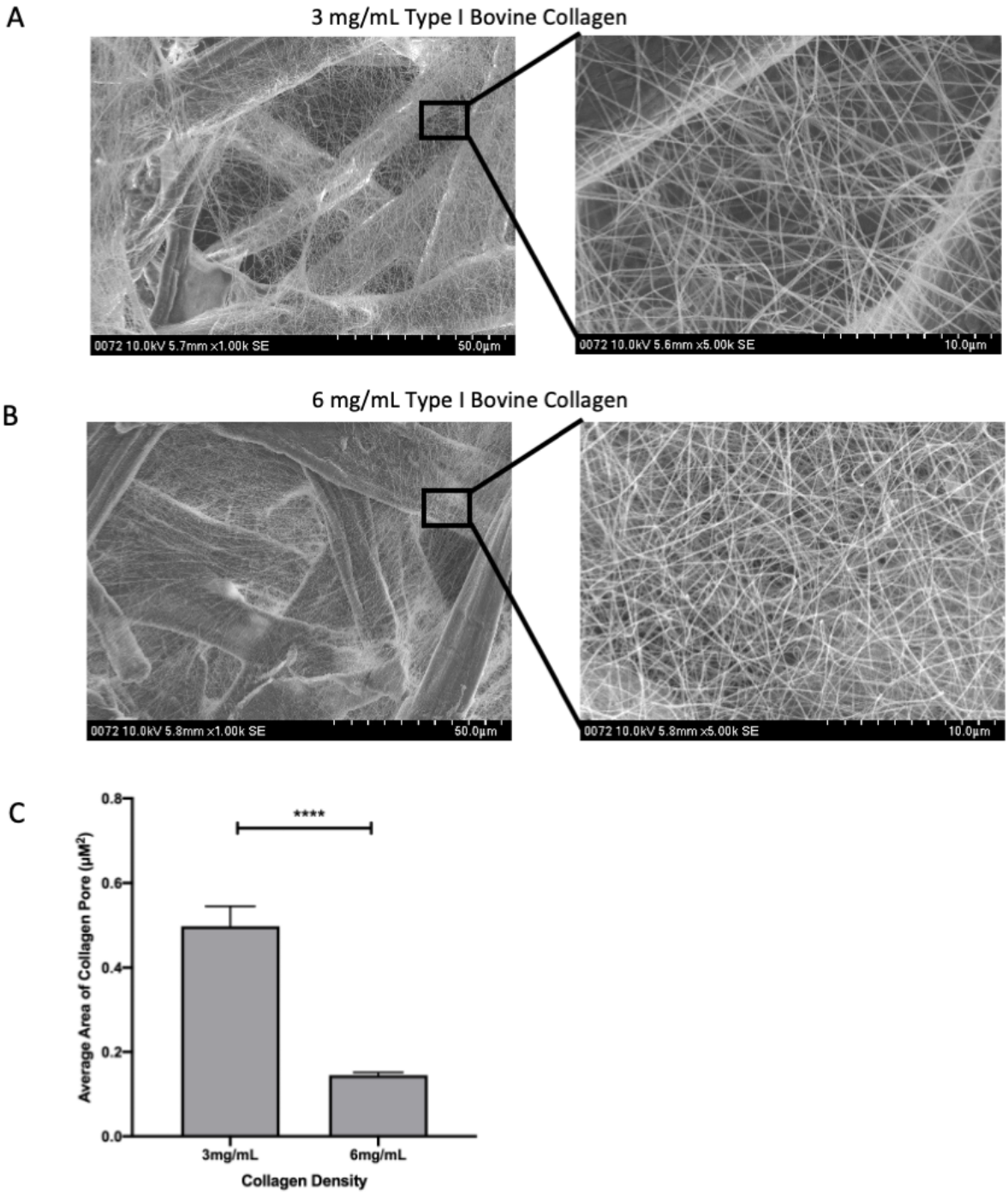
SI Figure 5. **CAFs derived from multiple patients promoted the movement of tumour cells into the central acellular collagen layer in TRACER**. Proportion of CAL33 tumour cells that moved into the central layer from the tumour seeded layer by 48h of rolled culture. TRACERs were fabricated at a seeding density of $15x10^6$ cells/mL in a collagen hydrogel of 6 mg/mL. Bars represent mean ± SEM of 3 independent experiments; statistical significance assessed by Student's T-test with a Bonferroni correction between monoculture and either mixed co-culture or segregated co-culture *** indicates $p<0.001$.



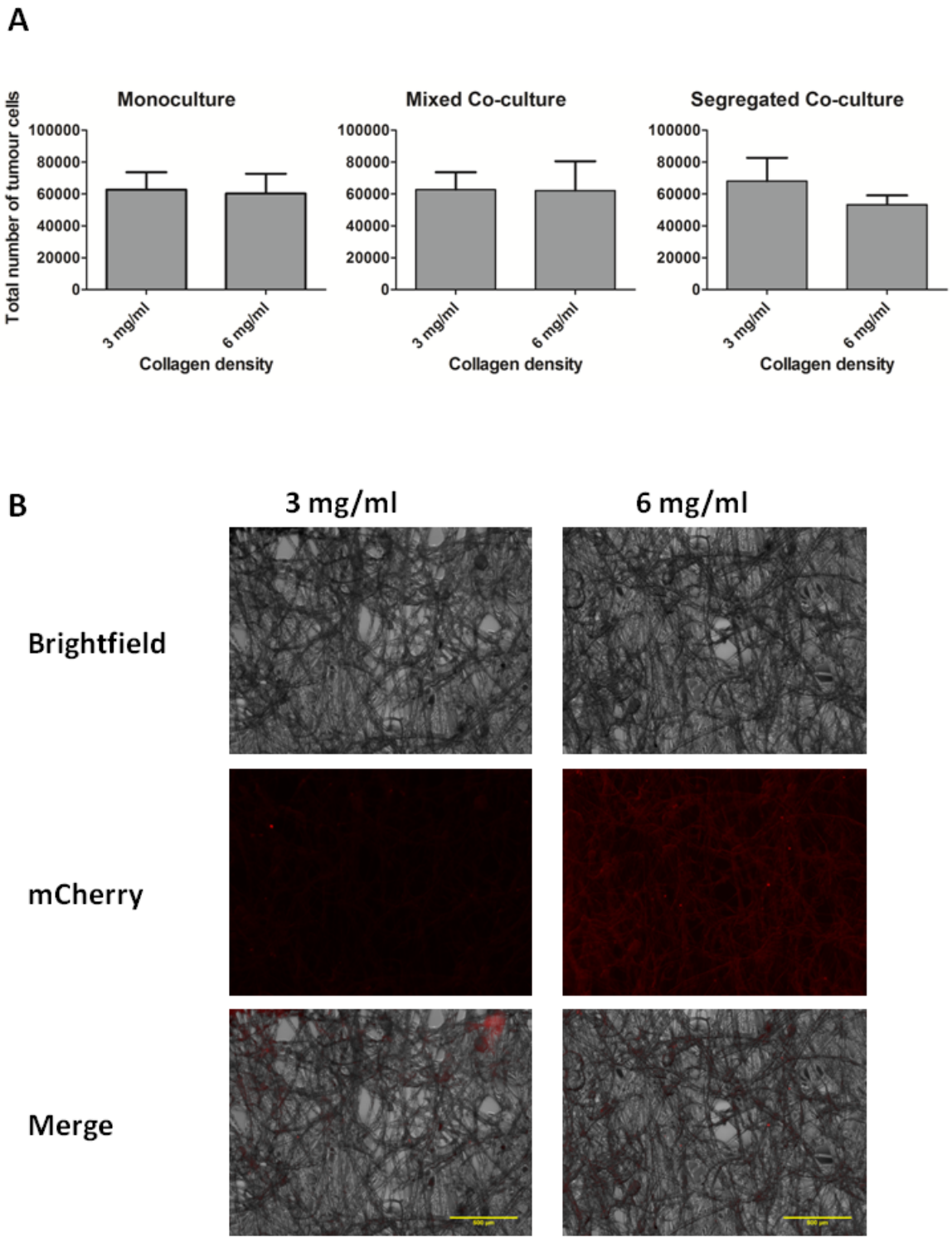
SI Figure 6. CAFs promoted the movement of tumour cells into the central acellular collagen TRACER layer in a reverse seeding configuration. Tumour cells were seeded in the outermost TRACER layer in the "reverse" configuration from standard experiments, Bar chart shows the fold change in the proportion of tumour cells that migrate into the central layer from the outer tumour seeded layer at 48h relative to monocultures. TRACERs were seeded at $15x10^6$ cells/mL in a 6 mg/mL collagen solution. Bars represent mean ± SEM of 3 independent experiments; Statistical significance between monoculture and either mixed co-culture or segregated co-culture was assessed using a Student's T-test with Bonferroni correction,* indicates $p<0.025$, ** indicates $p<0.005$.



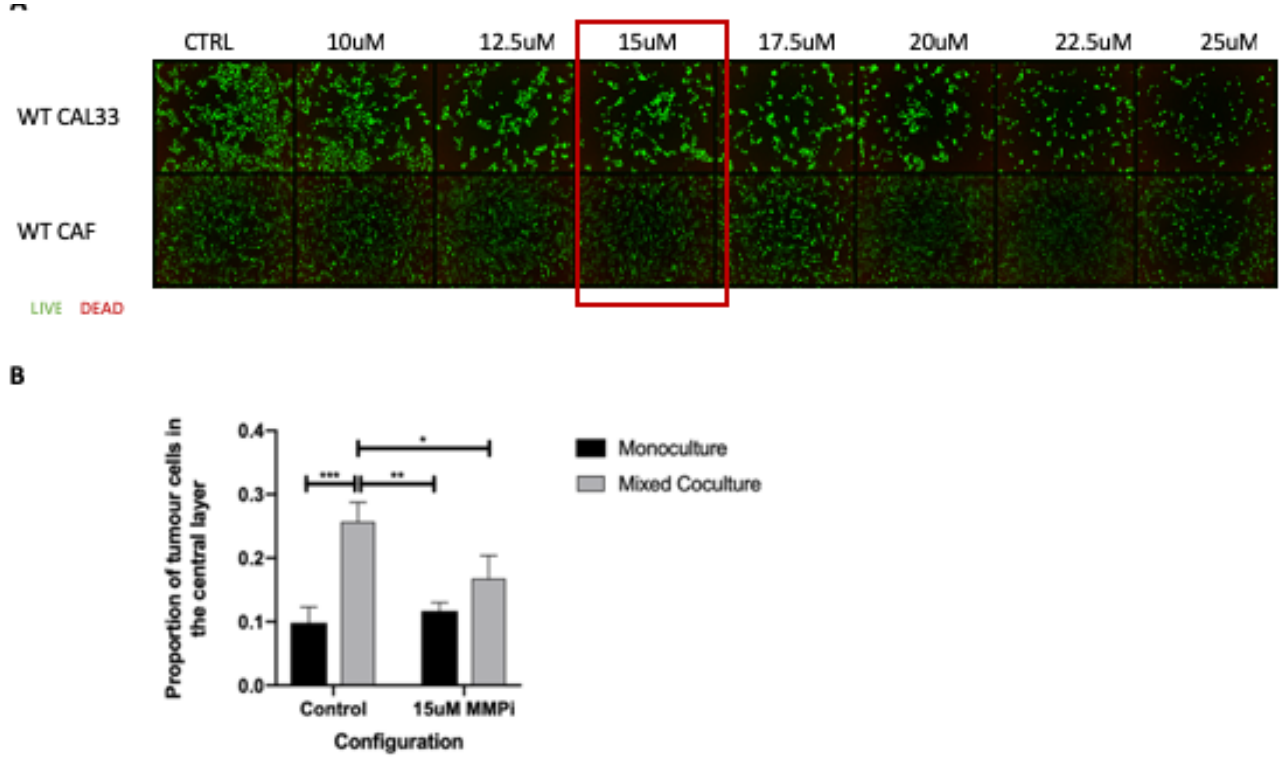
SI Figure 7. **The total number of tumour cells at 0h did not increase by 48h following treatment with mitomycin C**. Bar charts showing the total number of tumour cells from the innermost and central TRACER layers in monoculture, mixed co-culture, or segregated co-culture TRACERs in the presence of mitomycin C after 0h and 48h. TRACERs were fabricated at a seeding density of $30x10^6$ cells/mL in a collagen hydrogel of 6 mg/mL. Bars represent the mean±SEM of 3 independent experiments; Statistical significance between monoculture and either mixed co-culture or segregated co-culture was assessed using a Student's T-test with Bonferroni correction.



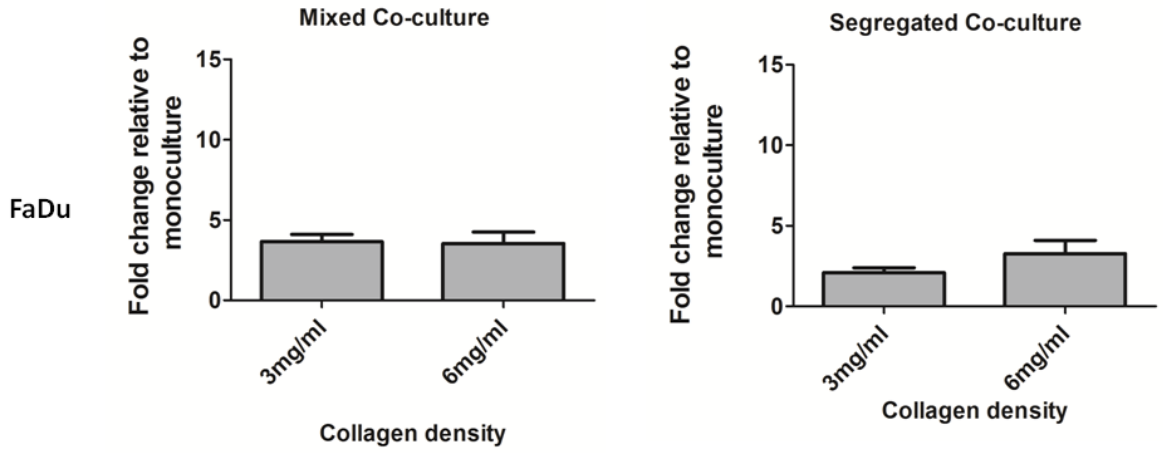
SI Figure 8: Assessment of porosity of 3mg/mL and 6mg/mL type 1 bovine collagen.
Paper scaffold was infiltrated with 3 mg/mL or 6 mg/mL type 1 bovine collagen hydrogel and porosity was measured by analysis of SEM images. A) Representative SEM image showing scaffold with visible paper fibers and 3 mg/mL collagen. B) Representative SEM images showing scaffold with visible paper fibers and 6 mg/mL collagen. C) Average pore sizes acquired from images from 3 images per sample of 3 samples (total 9 images) of each collagen density in 5K magnification images. Bars represent mean ± SEM; statistical significance was assessed using unpaired t-test, * indicates $p<0.033$, ** indicates $p<0.002$, *** indicates $p<0002$, **** indicates $p<0.0001$.



SI Figure 9: Digestion efficiency and extraction of cells from the TRACER scaffold was similar for both 6 mg/ml and 3 mg/ml collagen gels. A) Number of tumour cells digested from scaffold at 0h when scaffolds were fabricated using 6 mg/ml or 3 mg/ml collagen gel. Bars represent mean±SEM. Statistical significance was assessed using a Student's T-test with a Bonferroni correction. **B)** Representative images of scaffolds following digestion at 0h when scaffolds were fabricated using 6 mg/ml (left) or 3 mg/ml (right) collagen gel. Minimal cells (visible by the mCherry) signal remain in scaffolds after digestion. Scale bar is 500 µm.



SI Figure 10: CAF-enhanced CAL33 invasion is dependent on MMP activity.
A) CAL33 and CAF cell viability was assessed after 72h culture with the addition of 10μM to 25μM of GM6001 MMP inhibitor. Live (green) cells are labelled with calcein-AM and dead (red) cells are labelled with ethidium homidimer-1. No dead cells were observed at any concentration, but proliferation was decreased at higher inhibitor concentrations. A 15μM working concentration was therefore chosen for subsequent experiments. B) Proportion of CAL33 tumour cells that invaded into the central layer from the inner tumour seeded layer at 48h in TRACERs fabricated using 3 mg/mL hydrogels for both monoculture and mixed co-culture TRACERs, with and without addition of the MMP inhibitor. TRACERs were seeded at a density of $15x10^6$ cells/mL. Bars represent mean ± SEM of 3 independent experiments; statistical significance was assessed using Two-Way ANOVA with Turkey's post hoc test for multiple comparisons, * indicates $p<0.033$, ** indicates $p<0.002$, *** indicates $p<0002$.



SI Figure 11. Fold difference in FaDu tumor cell invasion at 48h in co-cultures relative to monocultures. CAF enhanced invasion of FaDu cells was similar in TRACERs fabricated using 3 mg/mL and 6 mg/mL collagen. Bars represent mean ±SEM for three independent experiments; Statistical significance was assessed using a Student's T-test.