Materials & Methods

Microfabrication of Silicon Moulds

A polished Si<100> wafer was cleaned in an ultrasonic bath using OptiClear (National Diagnostic), followed by acetone, methanol and then RO water (5 min in each). After priming by spin coating with Shipley Microposit primer (4000 rpm, 5 s), the wafer was coated with a layer of AZ4562 photoresist (4000 rpm, 30 s), which was then baked at 90°C on a vacuum hotplate for 5 min. The photoresist was exposed to UV light through a glass-emulsion mask (JD Photo-Tools) for 16 s using a SUSS MicroTec MA6 mask aligner and developed using a 1:4 dilution of AZ® 400K developer (in RO water) for approximately 3 minutes with gentle agitation. After ashing the wafer in a Gala Instruments PlasmaPrep5 barrel asher (2 min; 50 W; 0.2 mBar; 250 sccm O_2) to remove any residual photoresist from the developed areas, the exposed silicon was etched using a Bosch process in a STS (Surface Technology Systems) ICP DRIE system (28 min; 600/12 W (coil/platen); 32/20 mTorr (etching/passivation); 130/10 sccm SF_6/O_2 (etching) and 85 sccm C_4F_8 (passivation) with a 13s/7s etching/passivation cycle). The remaining photoresist was then stripped using acetone and the wafer cleaned in piranha solution (7:1 sulphuric acid/hydrogen peroxide) for 30 min before rinsing with RO water and blow-drying with nitrogen. In order to facilitate the removal of cured PDMS from the silicon mould, the clean wafer was silanised by immersion in a 1% perfluorooctyltrichlorosilane solution in ethanol for 30 min, followed by copious rinsing with ethanol, blow-drying with nitrogen and baking in a 120°C oven for 30 min.

Soft Lithography

The PDMS microcolumns were fabricated using Sylgard® 184 Silicone Elastomer kit. A 10:1 (w/w) ratio of the base and curing agent were thoroughly mixed together and poured over the silicon mould, which had been placed inside a Petri dish. The mixture was then degassed by placing the dish inside a vacuum desiccator and pumping down the chamber, before curing the PDMS overnight at 50°C. The cured PDMS was then gently peeled off the silicon mould.

Expression Vectors, Cell Culture & Lysis

The FRB fragment – the drug binding domain from the large PI3 kinase homologue FRAP/mTOR – from the pC4RHE plasmid (Ariad Pharmaceuticals Inc.) was transferred to the GST fusion vector pGEX-KG [1] using standard subcloning techniques. The GST-FRB fusion protein was expressed in *E. coli* BL-21s as follows. Bacteria from a stationary phase culture, grown overnight at 37ºC in a shaking incubator in LB broth with the antibiotics chloramphenicol and ampicillin, were diluted 1 in 100 into LB broth with antibiotics and grown at 37ºC to an OD_{500} of 0.6. IPTG was added to give a 1 mM concentration and the cells were grown for a further 3h at 37ºC. The bacteria were then pelleted by centrifugation (3220 x g for 30 min at 4ºC) and the pellet was frozen at -80ºC. After thawing to room temperature, the bacteria were resuspended in PBS supplemented with protease inhibitors (mini EDTA-free protease inhibitor tablets, Roche). Lysozyme was added to give 1 mg/ml and the bacterial suspension was incubated on ice for 30 minutes. The suspension was then subjected to 50 short pulses (~1 s) on ice using a probe sonicator (Branson Sonifier 150, setting 3). After centrifugation (3220 x g for 30 min at 4ºC), the supernatant was transferred to fresh tubes and frozen at -80ºC in small aliquots.

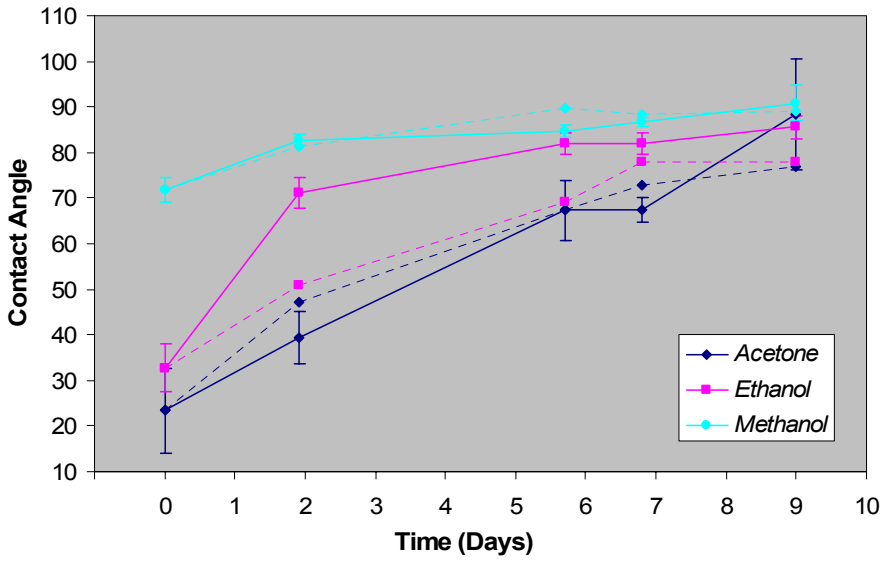
[1] K.L. Guan, and J.E. Dixon, “Eukaryotic proteins expressed in Escherichia coli: An improved thrombin cleavage and purification procedure of fusion proteins with glutathione-S-transferase”, *Analytical Biochemistry*, 1991, **192**, 262-267

Conventional Immunoprecipitation (IP)

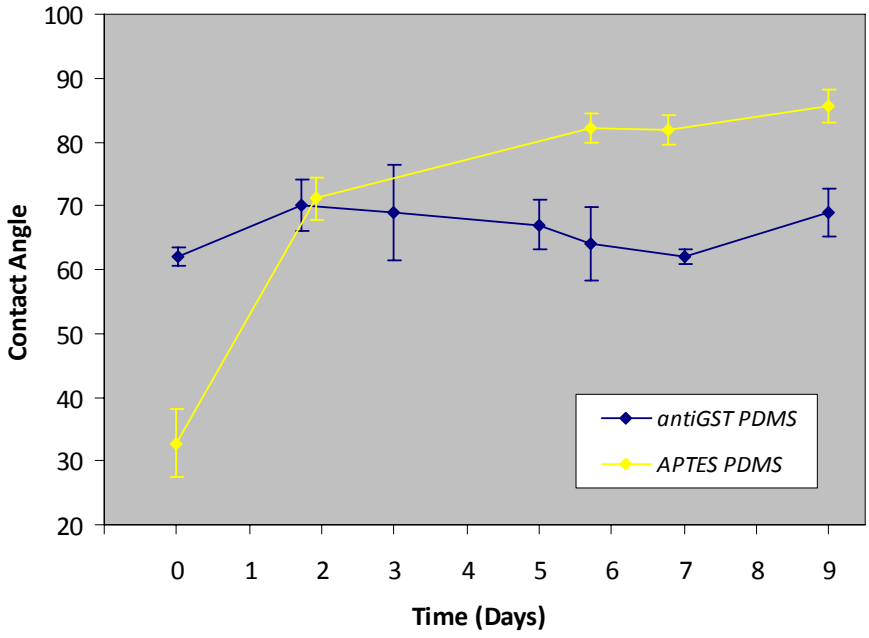
GST-FRB was immunoprecipitated from the *E. coli* lysate using antibodies immobilised on agarose resin as follows. 150 µl antiGST-agarose beads (Sigma Aldrich) was added to a LoBind microcentrifuge tube (Eppendorf) and washed 3 times using 0.1 M glycine pH 2.5, then 3 times with PBS by centrifugation at 100 x g for 2 min and discarding the supernatant. The lysate (300 µg total protein in 150 µl buffer) was incubated with the beads on an end-over-end mixer for 3 h at room temperature and then washed five times with 1 ml PBS.

Proteins were eluted using 50 µl of 10 mM reduced glutathione in 50 mM Tris, pH 8.0. The beads and glutathione were mixed for 5 min at room temperature before centrifuging at 100 x g for 2 min and collecting the supernatant. This elution process was repeated 3 times (i.e. four 50 µl elution fractions were collected). The eluted samples were run on a 10% SDS-PAGE gel and silver stained using the PlusOne™ kit (GE Healthcare) and visualised using a Syngene G:Box system.

S.I. Figure 1.



a.



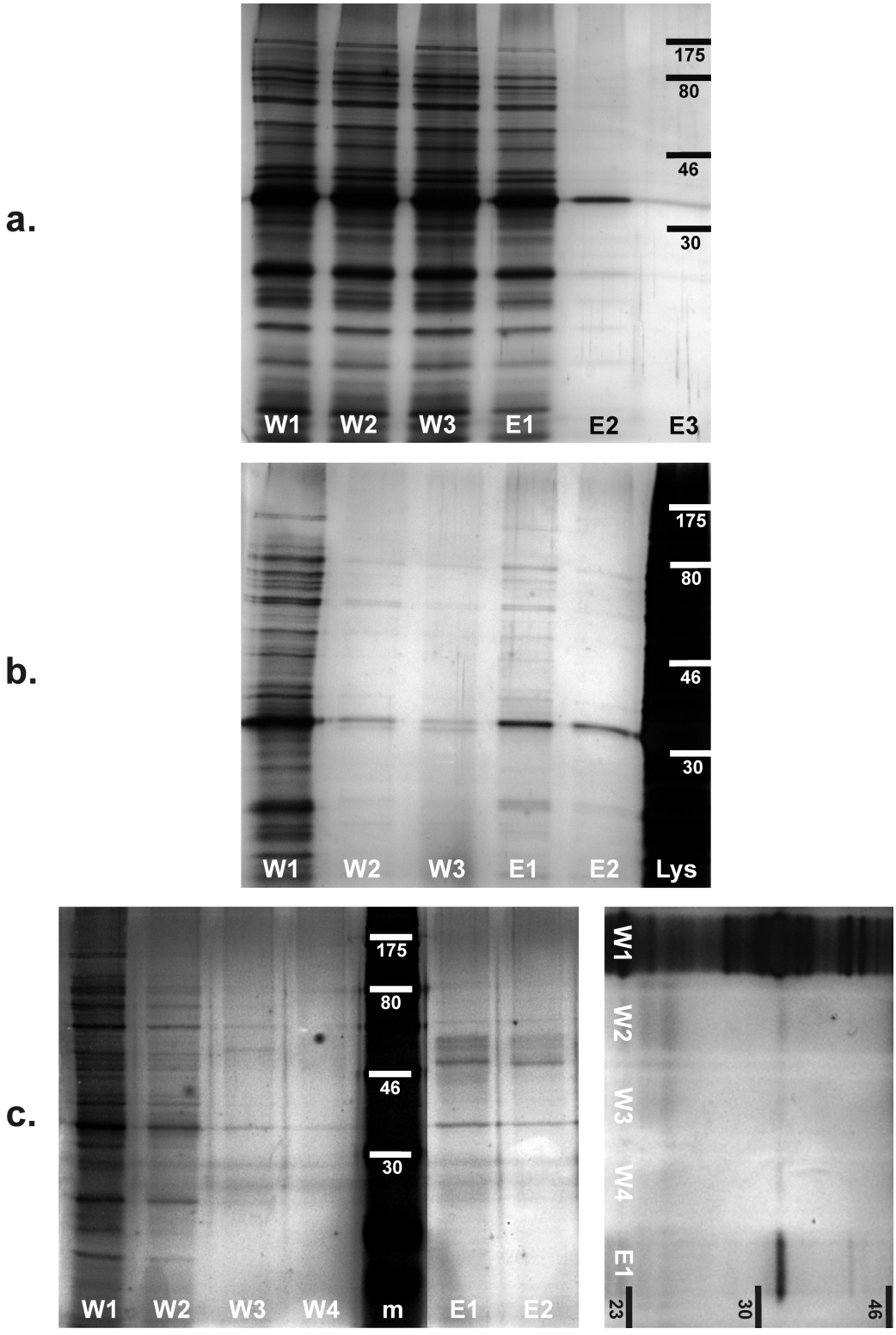
b.

Contact angle time courses of stored, functionalised PDMS. (a) Water contact angle measurements on PDMS functionalised with APTES following soaking of the PDMS in a either acetone or ethanol or cleaning in methanol as follows:

- acetone soak: immerse for 2 h in acetone, change solvent for fresh acetone, soak for a further 2h, then immerse in ethanol (1 h) and lastly methanol (1 h).
- ethanol soak: immerse for 2 h in ethanol, change solvent for fresh ethanol, soak for a further 2h, then immerse in methanol (1 h).
- methanol clean: sonicate in methanol for 15 min using an ultrasonic bath.

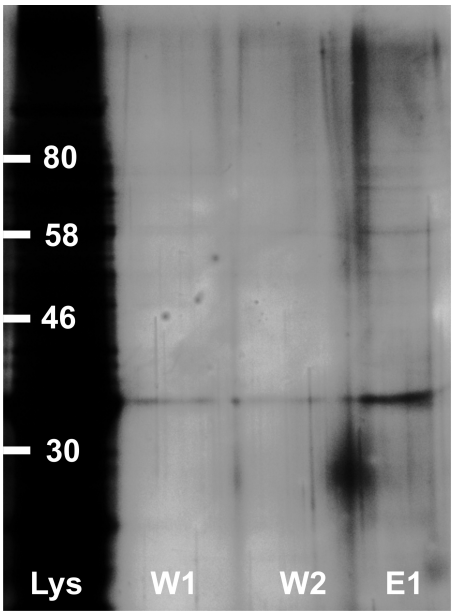
The solid line connects measurements acquired from a different sample at each time point (measurements were made at several points on the sample surface and error bars are one standard deviation), whilst the dashed line refers to measurements acquired from the same sample. (b) A comparison of the stability of APTES-functionalised PDMS (ethanol soak) with PDMS functionalised with antiGST (stored at 4°C in PBS-Azide).

S.I. Figure 2.



Silver stained gels from a series of GST-FRB (~37 kDa) µIP experiments, showing wash (W), elution (E) and lysate (Lys) fractions. Results from a 1:1 dilution (a), a 1 in 10 dilution (b) and a 1 in 20 dilution (c, left and right) of the bacterial lysate. The same wash, incubation and elution conditions were used for each experiment, with the exception of the second 1 in 20 dilution (c, right) which had both a longer sample incubation time and a smaller sample volume (10µl of diluted lysate and a 60 min total incubation, compared to 25µl diluted lysate and a 15 min incubation for the others).

S.I. Figure 3.



A silver stained gel showing wash (W) and elution (E) fractions from μIP of a bacterial lysate (1 in 20 dilution) containing GST-FRB (~37 kDa), using a microcolumn that had been stored for 7 days.