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A generic small-molecule microarray immobilization strategy

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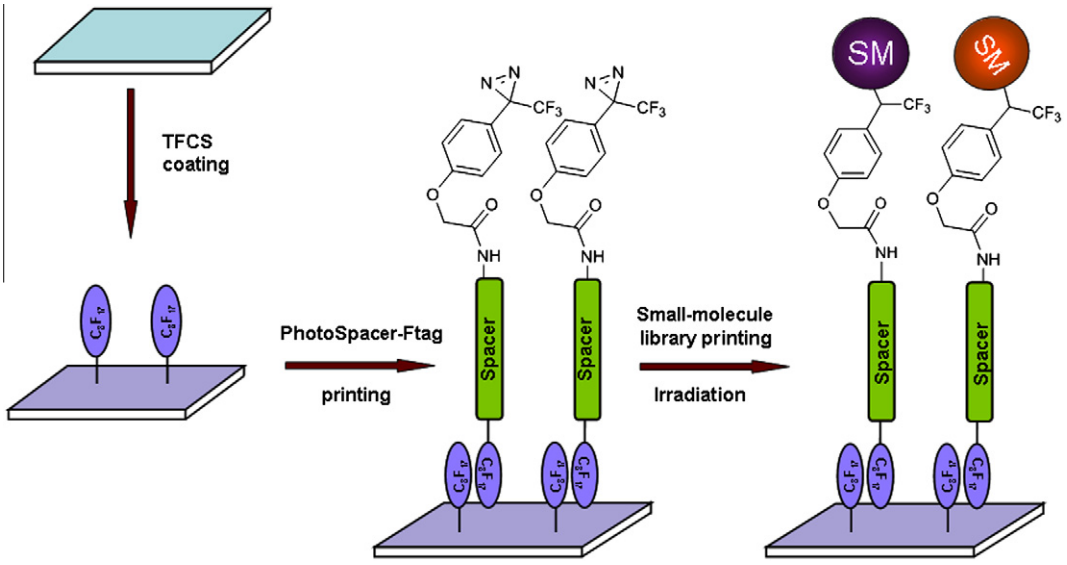
ABSTRACT

Small-molecule microarrays are often limited by the requirement for each compound undergoing immobilization to contain a common functional group or by the need to prepare glass slides containing photo-reactive groups. Herein, we present a generic strategy that allows any compound library to be immobilized. This was achieved by printing a fluorous-tagged photo-reactive 3-aryl-3-trifluoromethyldiazirine, which undergoes non-selective insertion into compounds following UV-activation, onto fluorous-functionalized glass slides. The arrays could be reused following aqueous stripping and re-assessment of the compounds with the same protein or another target of interest.

Small-molecule microarrays are potentially a powerful tool for the high-throughput screening of compound libraries with a variety of protein or cellular targets.[1] While small-molecule microarrays have allowed the identification of small molecule-protein binding partners,[2] this approach typically requires the inclusion of a specific common handle in the compounds of interest to facil-



Scheme 1. Generic small-molecule immobilization strategy. Fluorous slides were prepared by treating glass slides with tridecafluoro-1,1,2,2-tetrahydrooctyl-dimethoxy-chlorosilane (TFCS). Fluorous tagged diazirines were printed onto the fluorous slide with the small-molecules subsequently printed at the same location. Irradiation (350 nm) generates a carbene which inserts non-selectively into the over-laid compound.

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itate immobilization onto a functionalized glass slide.[3] One such approach has used fluorous tagging of the compounds with microarray fabrication on fluoroalkyl modified glass slides.[4] However, more common approaches are based on the generation of combinatorial libraries which are then covalently attached onto functionalized surfaces through a variety of selective chemical reactions such as 1,4-additions, Diels–Alder, Staudinger or 1,3-dipolar addition chemistry or biotin/avidin interactions.[5] A generic approach for the stable capture of compounds has been the application of photoreactive surfaces prepared from diazirines and aryl azides which after irradiation generate highly reactive groups which undergoes non-selective insertion reactions into a broad range of molecules.[6]

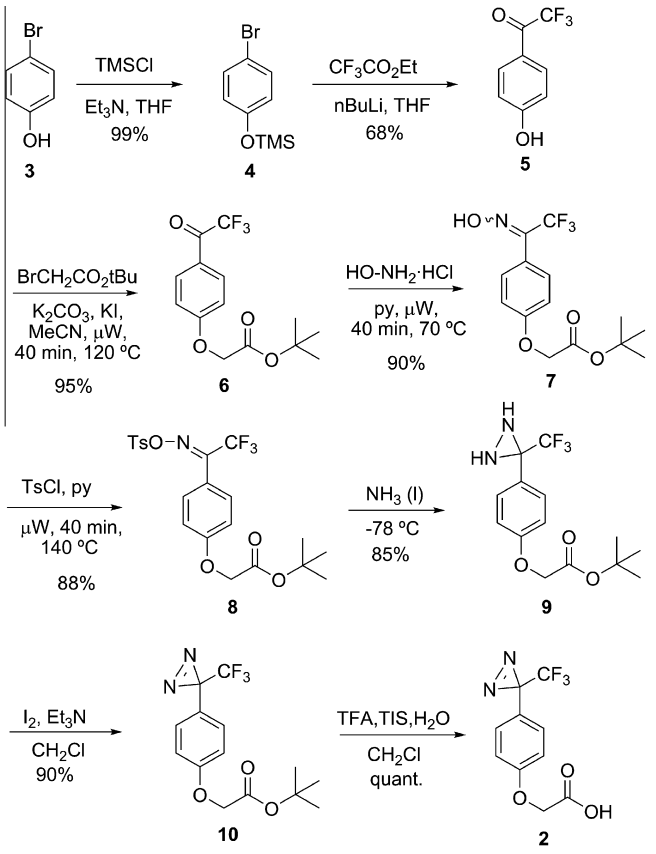
Here, we describe the development of a generic method for small-molecule immobilization which combines two strategies, the covalent capture by photo-crosslinking through a diazirine functional group tied in with the advantages of non-covalent fluorous affinity interactions (Scheme 1). The photochemistry immobilization approach allows the attachment of any type of compound library without the need of library synthesis to include a common handle, while widening the scope of the technique to include natural products; while fluorous chemistry prevents non-specific interactions with proteins or cells, lowering the background and increasing the signal-to-noise ratios. In addition, this hybrid method offers the possibility of recovering individual spots for further analysis and protein stripping allowing recycling/reuse of the array.

The strategy consisted of the immobilization of a fluorous tagged diazirine on a fluorous surface. Irradiation of the diazirine generates a carbene which immobilizes any proximal small molecule in a non-selective manner. Experiments for the validation and optimization of the approach were carried out using fluorescein and biotin as model small molecules.

As the length of the spacer (see Scheme 1) would be expected to have an effect on immobilization efficiency[7] three different photospacers were synthesized by solid phase synthesis using the Fmoc-protected spacer unit (Fmoc-8-amino-3,6-dioxaoctanoic acid spacer **1**) and the photoreactive diazirine derivative {4-[3-(trifluoromethyl)-3*H*-diaziren-3-yl]phenoxy}acetic acid (**2**).

The synthesis of the diazirine derivative **2** started with the protection of 4-bromophenol (**3**) with trimethylsilyl chloride followed by a reaction with *n*BuLi and trifluoromethyl ethyl acetate to give **5** in 68% yield (Scheme 2). The phenol was then alkylated with *t*-butyl bromoacetate under microwave irradiation to give **6**. Oxime **7** was generated by treatment with hydroxylamine hydrochloride in pyridine and reacted with *p*-toluensulfonyl chloride to give **8**. Both reactions were performed under microwave irradiation. Formation of diazirine **9** was achieved by treatment of **8** with liquid ammonia overnight. Iodine was used for the oxidation of **9** to give, following removal of the *t*-butyl protecting group with trifluoroacetic acid, the trifluoromethyl aryldiazirine **2** in an overall yield of 35%. Reaction times for some of the steps were shortened by using microwave-assisted reactions.[8b]

The fluorous tagged photoreactive spacers were synthesized from resin **11** (Scheme 3) which was esterified with **1** to give **12**. This was treated with 20% piperidine and the free amine was coupled to {4-[3-(trifluoromethyl)-3*H*-diaziren-3-yl]phenoxy}acetic acid (**2**) to give **14**. The desired product **15** was obtained by cleavage from the resin with a standard acidic cocktail (48% TFA, 48% CH_2Cl_2, 2% water, and 2% TIS).[8] Two related constructs **16** and **17** were synthesized by the repeated addition of the Fmoc-8-amino-3,6-dioxaoctanoic acid spacer **1** before capping with TFMAD and cleavage (Scheme 3). The fluorous tagged photoreactive spacers were synthesized by coupling **15**, **16** and **17** to 1-amino-



Scheme 2. Synthesis of {4-[3-(trifluoromethyl)-3*H*-diaziren-3-yl]phenoxy}acetic acid (TFMAD) (**2**).

1*H*,1*H*,2*H*,2*H*-perfluorodecane, using PyBop and DIEA, and purified *via* a fluorous silica column to give **18**, **19**, and **20**.

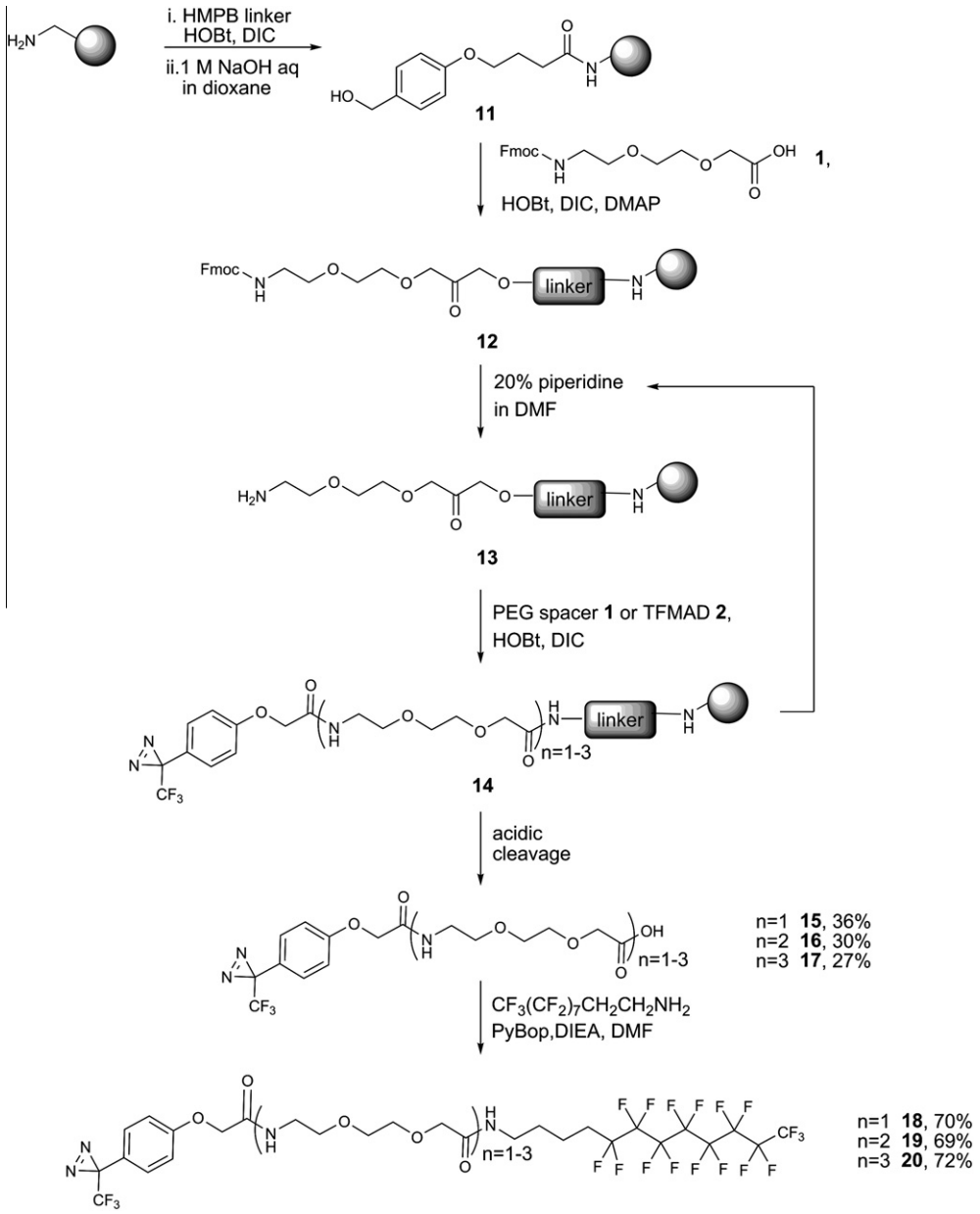
Fluorous tagged biotin **21** and carboxyfluorescein **22** were synthesized as positive controls in the same manner (Fig. 1).

In order to establish the best approach to immobilize compounds different strategies for printing and washing the slides were assessed using fluorous tagged carboxyfluorescein **22**. Following printing, it was observed that the slides could be washed with water without fear of compound diffusion, but any organic solvent completely removed the immobilized compounds from the slides.[8]

To determine the optimal photoreactive spacer length, compounds **18**, **19** and **20** were printed on the fluorous slides followed by a solution of fluorescein. The spots were dried and irradiated for 40 min at 365 nm to generate the carbene and enable fluorescein immobilization (Fig. 2).

This demonstrated that the shortest fluorous tagged photoreactive spacer **18** gave the best results with the most intensive signals with all concentrations of the dye. It also demonstrated that the diazirine photoreactive spacer could capture small molecules and display good sensitivity and signal-to-noise ratios with the printing of 10 mM solutions, easily achievable with compound library collections.

Photoreactive spacer **18** (5 × 5 spots) was used to immobilize biotin with optimization of the photoreactive spacer/biotin ratio. The presence of biotin covalently attached to the surface was confirmed by binding with Cy3-labeled Streptavidin (Fig. 3). This showed that



Scheme 3. Synthesis of the photoreactive fluorous spacers by solid phase synthesis.

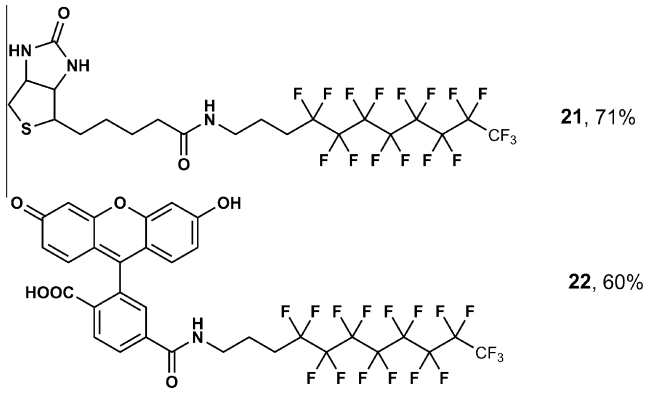


Figure 1. Positive controls prepared for the fabrication and optimization of the fluorous-tagged microarrays.

biotin was immobilized successfully and that a large excess of photoreactive spacer did not improve signal-to-noise ratios, with the best ratios being 1:1 and 1:2 (photoreactive spacer/biotin).

The Streptavidin-Cy3 incubated slides could be reused. Thus the slides could be stripped by treating with water at 70 °C for 2 h and re-incubated with Cy3-Streptavidin, demonstrating efficient re-binding (Fig. 4).

This approach, making use of carbene insertion chemistry, simplifies small-molecule immobilization making it suitable for arraying and screening libraries of drug-like small molecules arrayed on fluorous coated glass slides. The fluorous surface also has advantages of avoiding non-specific interactions with proteins and cells. Furthermore the use of fluorous slides allows the recycling of the slides either by total stripping (organic solvent) or removal of the interrogation protein (water treatment at 70 °C) and reuse for repeated screening. We have also shown that a large excess of the photoreactive spacer is not translated to better immobilization and that the smaller spacer length is important for high ligand binding and sensitivity of detection.

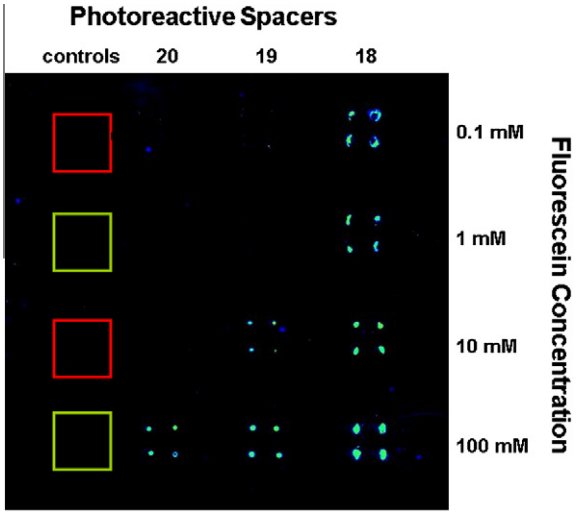


Figure 2. Photoreactive fluorous spacer optimization. Photoreactive spacers **18**, **19** and **20** were contact printed (10 mM solution in DMF) and allowed to dry. Subsequently, different concentrations of carboxyfluorescein in DMF were printed onto the photoreactive spacers and the slides were left to dry overnight. Irradiation of the slide was carried out for 40 min at 365 nm before washing and scanning. Negative controls (highlighted with green and red boxes) correspond to the printing of the spacer **18** or unmodified carboxyfluorescein, respectively.

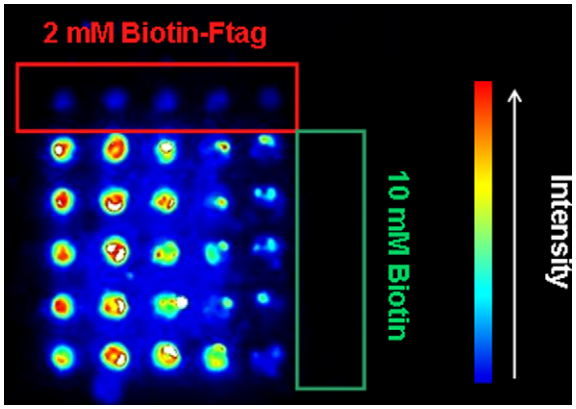


Figure 3. An inkjet printer was used to fabricate the array. The photospacer **18** was used for the immobilization of a solution of 10 mM biotin. Columns (from left to right) represent various photospacer/biotin ratios (1:1, 2:1, 3:1, 4:1, and 5:1) with each row a series of replicates. The green rectangle represents spots of 10 mM biotin solution printed without the photospacer (negative control). The red rectangle represents printing of fluorous tagged biotin (**21**) (2 mM) (positive control). The slides were left to dry and then irradiated for 40 min at 365 nm and washed extensively before incubation with Streptavidin-Cy3 for 1 h at 37 °C and scanned with a Cy3 filter.

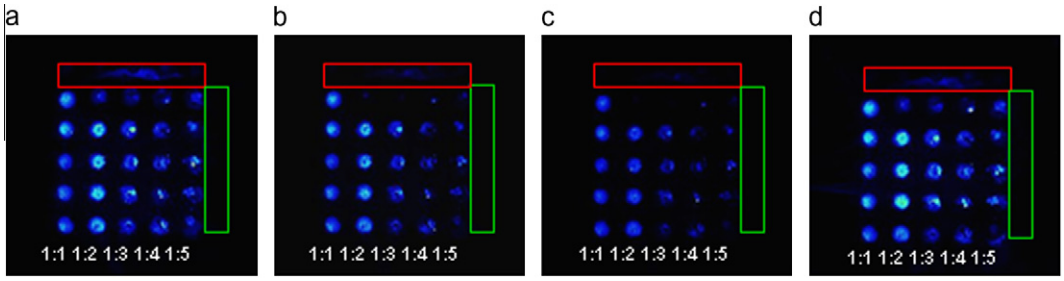


Figure 4. Slide recycling: (a) Initial incubation with Streptavidin-Cy3; (b) washing for 1 h at 70 °C; (c) washing for 2 h at 70 °C; and, (d) re-incubation with Streptavidin-Cy3.

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