Nanoliter Plates – Versatile Tools for the Screening of Split-and-Mix Libraries On-bead and Off-bead

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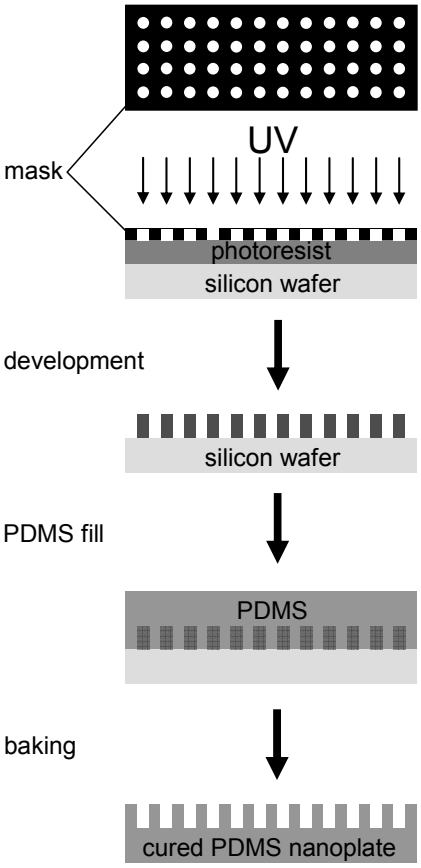
1. General aspects and material

Materials and reagents were of the highest commercially available grade and used without further purification. Enzymes, organic materials and solvents were purchased from Fluka and Sigma-Aldrich, amino acids from Bachem. Tentagel (90µm) was purchased from Rapp Polymers (Tübingen, Germany). For all the solutions and the washing steps MilliQ double distillated water was used. The irradiation was carried out using a 100W UV lamp (Black-Ray B-100AP). Nanoliter plate assays were analyzed with an Olympus SZX12 microscope. GC analyses were performed on a Hewlett Packard HP 6890 from Agilent.

2. Fabrication of the nanoliter plates

The nanoliter plates were fabricated by patterning wells with a depth of 225 µm and a diameter of 150 µm into poly(dimethylsiloxane) (PDMS) using soft-lithography (Figure 1. Fabrication of nanoplates. A photomask is generated using a computer animated design (CAD) program. Subsequently the pattern is projected onto a silicon wafer coated with photoresist using UV light. Specific polymerization of the exposed areas results in a mold that can be filled with PDMS. After baking, the cured PDMS is peeled off and can optionally be rendered hydrophilic by plasma treatment.).[1] In brief, a mould of SU-8 resist (MicroChem Corp. SU-8 2100) was fabricated on a silicon wafer (Siltronix) by UV exposure (MJB3 contact mask aligner; SUSS MicroTec) through a photolithography mask (Selba SA, Switzerland) and subsequent development (SU-8 developer; MicroChem Corp.). A curing agent was added to the PDMS base (Sylgard 184 silicone elastomer kit; Dow Corning Corporation) to a final concentration of 10% (w/w), mixed and poured over the mould to a depth of 5 mm. After degassing the mixture for several minutes, cross-linking was performed at 65 °C for several hours. Subsequently, the PDMS was peeled off the mould and rendered hydrophilic by incubation for 3 minutes in oxygen plasma (Plasma Prep 2, Gala Instrument).



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3. General protocol for the preparation of beads with photocleavable linker

i-Pr_2NEt (76 µL, 0.432 mmol) was added to a solution of 4-(4-nitro-3-((tetrahydro-2*H*-pyran-2-yloxy)methyl)phenyl)butanoic acid (73 mg, 0.216 mmol)[2] and HCTU (89 mg, 0.216 mmol) in DMF. The activated amino acid was added to the amino-functionalized Tentagel resin (200 mg, 0.054 mmol) preswollen in DMF (~100 mM concentration) and the mixture was agitated for 1.5 h before washing with DMF and CH_2Cl_2 (5x each). The coupling efficiency was checked by the Kaiser test. Dioxane saturated with HCl was added to the resin (pre-swollen in dioxane) and the reaction mixture was agitated for 1.5 h. The resin was then washed with DMF and CH_2Cl_2 (5x each) and dried under vacuum.

4. Filling procedure and statistic

A solution of acetylated Tentagel NH_2 beads (Ø=90 µm, Rapp Polymer) pre-swollen in water (100 mg/mL, ~50 µL/cm^2) was spread on the wet nanoliter plate with a spatula. The surplus was flushed using compressed air (axis flux at 45° with the nanoliter plate). A Methyl Red Sodium solution (1 mg/mL, ~50 µL/cm^2) was added and the surplus was flushed with air. Mineral oil (~100 µL/cm^2) was added to seal the wells. Reproducible filling efficiencies of 90±5 % of all the wells with single bead were achieved.

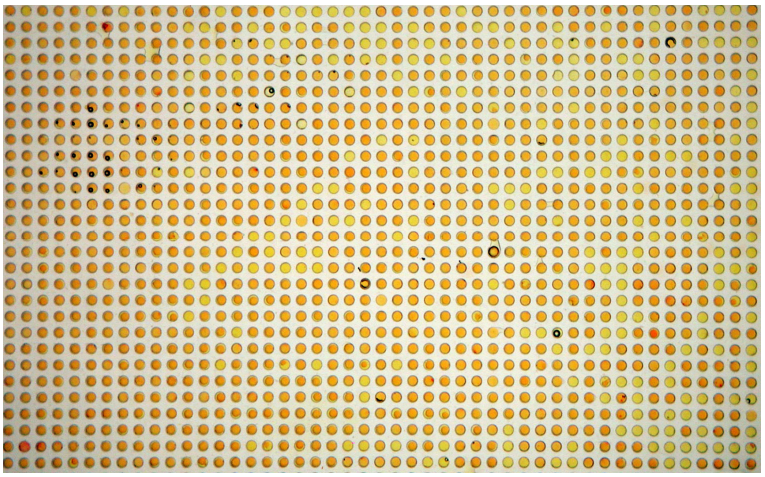
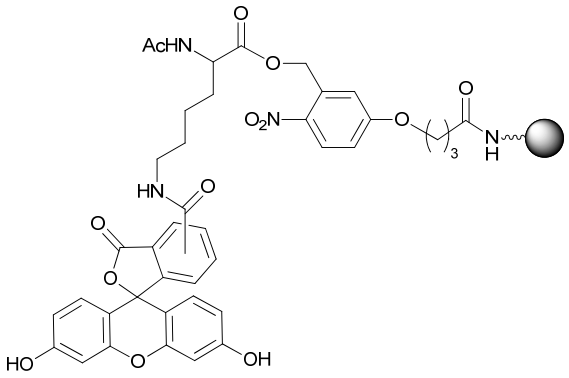


Figure 2. Nanoliter plate filled with acetylated Tentagel beads and coloration with Methyl Red solution. Red/orange wells contain beads (89%) whereas yellow wells are empty (11%). The red colour is due to the non-specific adsorbtion of the hydrophobic dye to the beads.

5. Cross-contamination studies

5.1. Preparation of fluorescein derivative beads.



Under nitrogen atmosphere, MSNT (90 mg, 0.270 mmol) was added to Fmoc-Lys(Boc)-OH (128 mg, 0.270 mmol) and 1-methylimidazole (15 μL, 0.189 mmol) in dry CH_2Cl_2. The activated amino acid was added to Tentagel resin (200 mg, 0.054 mmol) functionalized with the photocleavable linker functionalized preswollen in CH_2Cl_2 (~100 mM concentration) and the mixture was agitated for 1.5 h before washing with DMF and CH_2Cl_2 (5x each). The reaction was repeated to ensure complete coupling. 20% v/v piperidine in DMF was added to the resin (pre-swollen in DMF) and the reaction mixture was agitated for 5 min, drained and the piperidine treatment repeated for 10 min. The resin was then washed with DMF and CH_2Cl_2 (5x each). A solution of Ac_2O/TEA/DMF (1/1/8) (1 mL) was added. After 15 min, the beads were washed with DMF and CH_2Cl_2 (5x each). A solution of TFA/Trisopropylsilane (TIS)/CH_2Cl_2 (8/0.5/1.5) was added and after 15 min, the resin was washed with DMF and CH_2Cl_2 (5x each). *i*-Pr_2NEt (76 μL, 0.432 mmol) was added to a solution of 5(6)-carboxyfluorescein (81 mg, 0.216 mmol) and HCTU (89 mg, 0.216 mmol) in DMF. The activated amino acid was added to the resin. After 1.5 h, the beads were washed successively with a solution 20% v/v piperidine in DMF (3x), DMF (5x) and CH_2Cl_2 (5x) and dried under vacuum. All coupling and deprotection steps were checked by Kaiser tests.

5.2. Cross-contamination study

Nanoliter plates were filled as described above (page S3) with a mix of acetylated and fluorescein derivatized beads (ratio 1/1). After sealing of the wells with mineral oil, the plate was irradiated during 5 min with an UV lamp (365 nm).

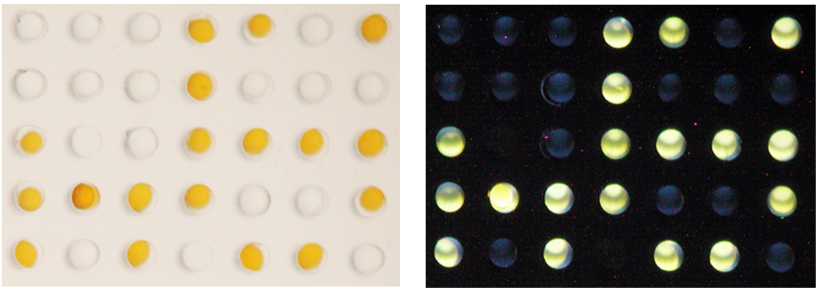


Figure 3. Nanoliter plate filled with acetylated and fluorescein derivative beads after 5 min upon UV irradiation.

6. Phosphate and acetal hydrolysis

6.1. Preparation of the enzyme grafted beads

500 µL of a solution of glutaraldehyde (2.5 % v/v in water) were added to 50 mg of Tentagel NH_2 beads pre-swelled in water. After 5 min, the beads were washed 3 times with buffer (Phosphatase buffer: HEPES 0.1 M pH=7, β-D-Galactosidase buffer: Na_2PO_4 0.1 M pH=7). 250 µL of a solution of enzyme in buffer (1 mg/mL) were added to the resin. After 3 h, the beads were washed 5 times with buffer and their enzyme activity were examined by addition of their respective substrates fluorescein diphosphate (FDP) or fluorescein di(β-D-galactopyranoside) (FDGal) to 1 mg of resin.

6.2. Test for hydrolytic activity

Nanoliter plates were filled as described above (page S4) with a mix of acetylated beads and enzyme derivatized beads (ratio 5:1). Substrate solution (0.1 mg/mL) (fluorescein diphosphate (FDP) or di(β-D-galactopyranoside) (FDGal)) was then added, spread and flushed quickly. Wells were sealed with mineral oil. After 15 min, pictures at visible and fluorescent light were recorded (Figure 4 and Figure 5).

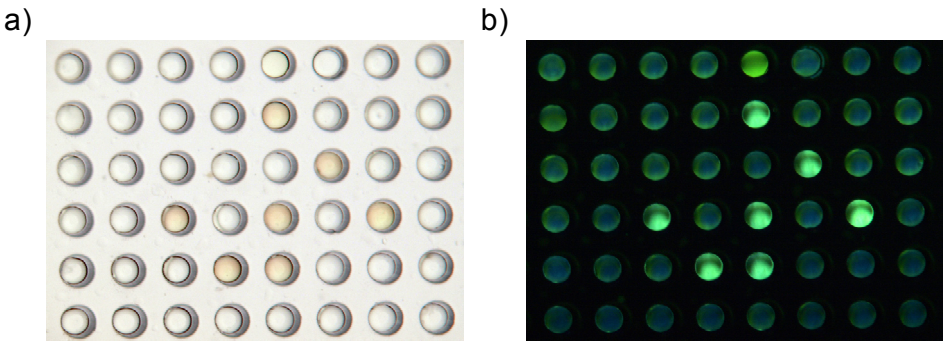


Figure 4. Nanoliter plates filled with phosphatase grafted beads (slighly brown under visible light) and acetylated beads with FDP (0.1 mg/mL).

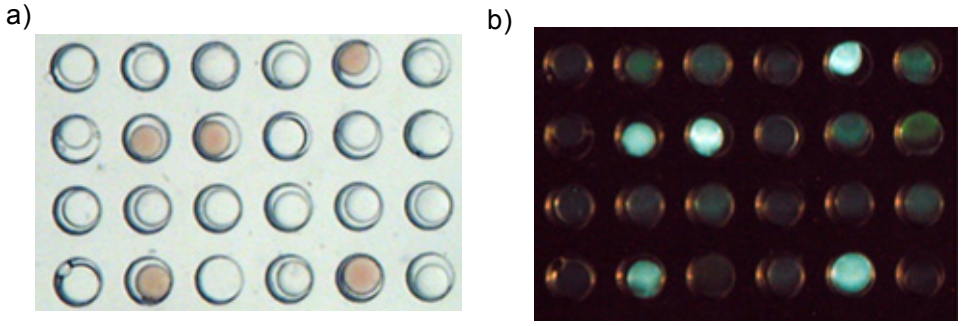
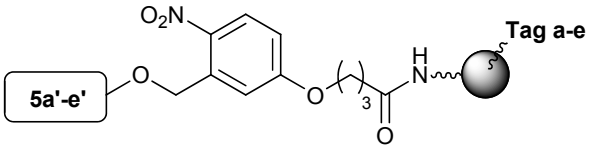


Figure 5. Nanoliter plates filled with galactosidase grafted beads (slighly brown under visible light) and acetylated beads with FDGal (0.1 mg/mL).

7. Enzyme-inhibitor model studies

7.1. Preparation of immobilized compounds 5a-e.



For each compound **5a-e**, 100 mg of Tentagel-NH_2 (0.03 mmol) were encoded using 5 mol % of unique oxidatively cleavable tags for each compound.[3] The photocleavable linker was then coupled and deprotected following the procedure described previously (page S3). Each compound was then coupled using the MSNT/1-methylimidazole procedure.

Compound **5a'**:

Under nitrogen atmosphere, MSNT (44 mg, 0.150 mmol) was added to Boc-4-Abz-OH (36 mg, 0.150 mmol) and 1-methylimidazole (15 µL, 0.105 mmol) in dry CH_2Cl_2. The activated amino acid was added to the amino-functionalized Tentagel resin (100 mg, 0.030 mmol) preswollen in CH_2Cl_2 (~100 mM concentration) and the mixture was agitated for 1.5 h before washing with DMF and CH_2Cl_2 (5x each). The reaction was repeated a to ensure complete coupling. The Boc protective group was then removed by treatment with TFA/CH_2Cl_2/TIS (8/1.5/0.5) during 15 min and the resin was then washed with CH_2Cl_2 and DMF (5x each) followed by 5 times washing with buffer Tris-HCl 0.1M pH=7.

Compound **5b'**:

Same procedure as described for compound **5a'** but with Boc-2-Abz-OH (36 mg, 0.150 mmol).

Compound **5c'**:

Same procedure as described for compound **5a'** but without TFA treatment.

Compound **5d'**:

Same procedure as described for compound **5a'** but with benzoic acid (19 mg, 0.150 mmol) and without TFA treatment.

Compound **5e'**:

Same procedure as described for compound **5a'** but with Fmoc-Phe-OH (58 mg, 0.150 mmol) and Fmoc removal using 20% v/v Piperidine/DMF solution.

7.2. Filling procedure and bead analysis

A mixture of the differently functionalized beads (5 mg of each resin) were mixed with 250 µL of a solution of Horseradish peroxidase (0.1 mg/mL) in Tris-HCl buffer at pH=7. Approximatively 100 µL of the bead suspension were spread on a nanoliter plate (~2 cm^2) and the surplus was removed by an air flux. The plate was irradiated for 2 min with a UV lamp (365 nm) and 100 µL of a solution of 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (40 mg/mL) were added, spread and flushed quickly. H_2O_2 solution (0.5 M) was added

in the same way and the wells were seal using mineral oil. After 10 min, beads localized in uncolored and colored wells were isolated using a sharp and small spatula (Figure 6a). For decoding, the tags were cleaved and analyzed by GC/ECD.[3]

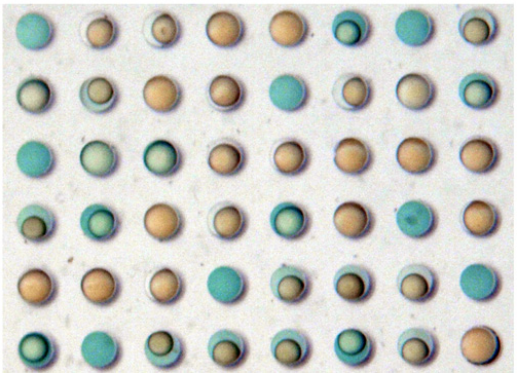


Figure 6. Nanoliter plates with immobilized compounds **5a-e** with UV release.

References

1. T.M. Squires, S.R. Quake, *Rev. Mod. Phys.* 2005, **77**, 977.
2. J.C. Lee, C.Y. Wu, J.V. Apon, G. Siuzdak, C.H. Wong, *Angew. Chem. Int. Ed.* 2006, **45**, 2753.
3. H. P. Nestler, P. Bartlett, W.C. Still, *J. Org. Chem.* 1994, **59**, 4723.