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Detergent screening of a G-protein-coupled receptor using serial and array biosensor technologies

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ABSTRACT

We describe the benefits and limitations of two biosensor approaches for screening solubilization conditions for G-protein-coupled receptors (GPCRs). Assays designed for a serial processing instrument (Biacore 2000/3000/T100) and an array platform (Biacore Flexchip) were used to examine how effectively 96 different detergents solubilized the chemokine receptor CCR5 while maintaining its binding activity for a conformationally sensitive Fab (2D7). Using the serial processing instrument, we were able to analyze three samples in each 30-min binding cycle, thereby requiring approximately 24 h to screen an entire 96-well plate of conditions. In-line capturing allowed us to normalize the 2D7 binding responses for different receptor capture levels. In contrast, with the array system, we could characterize the effects of all 96 detergents simultaneously, completing the assay in less than 1 h. But the current array technology requires that we capture the GPCR preparations off-line, making it more challenging to normalize for receptor capture levels. Also, the array platform is less sensitive than the serial platforms, thereby limiting the size of the analyte to larger molecules (>5000 Da). Overall, the two approaches proved to be highly complementary; both assays identified identical detergents that produced active solubilized CCR5 as well as those detergents that either were ineffective solubilizers or inactivated the receptor.

Surface plasmon resonance (SPR)[1] biosensors can be used to identify suitable conditions for isolating G-protein-coupled receptors (GPCRs) from membranes while preserving the receptors' ability to bind conformationally sensitive ligands. For example, we have used SPR in the past to screen GPCR solubilization, purification, and crystallization conditions [1–3]. We identified components of solubilization and crystallization buffers that increased a GPCR's stability, and we optimized affinity purification methods to yield highly active receptor.

Here we outline two biosensor-based assays that reveal how different detergents affect the yield and activity of solubilized GPCRs. We used CCR5 as the model system because we have access to high-quality reagents and binding partners. Our CCR5 construct is C-terminally tagged with a peptide sequence recognized by 1D4 antibody [4], which stably captures the receptor yet can be regenerated at the end of each binding cycle. To probe the conformation of CCR5, we used a Fab of the 2D7 antibody.

The detergent screening methods were developed using Biacore 2000 and Flexchip platforms. The microfluidics system in the Biacore 2000 can test one analyte across a reference and up to three reaction surfaces [5]. After each binding cycle, the surfaces are regenerated and new aliquots of receptor are captured. Although serial sample processing decreases throughput, this instrument's sensitivity (it is routinely used to examine molecules <500 Da), combined with its ability to monitor both ligand capture and analyte binding, makes it useful for receptor screening. In the Biacore Flexchip array platform, an analyte is flowed across a matrix of ligand "spots" within a single large-format flow cell, and this can significantly improve ligand throughput [5,6]. But array biosensors have lower sensitivity than traditional serial platforms; Flexchip's limit of detection is approximately 5000 Da. In addition, ligands currently are spotted off-line, and so capture levels, for example, cannot be quantitated directly.

Our screening methods took advantage of the unique features of these two platforms. With Biacore 2000, we established the corre-

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[1] *Abbreviations used:* SPR, surface plasmon resonance; GPCR, G-protein-coupled receptor; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt); PEG, polyethylene glycol; mAb, monoclonal antibody; EDTA, ethylenediaminetetraacetic acid; HBS, Hepes-buffered saline; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); RU, resonance units; CHS, cholesteryl hemisuccinate Tris salt; Chaps, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate/*N*,*N*-dimethyl-3-sulfo-*N*-[3-([(3α,5β,7α,12α)-3,7,12-trihydroxy-24-oxocholan-24-yl]amino)propyl]-1-propanaminium hydroxide, inner salt; DDM, *n*-dodecyl-β-D-maltopyranoside; BSA, bovine serum albumin; OGPS, *n*-octyl-β-D-glucopyranoside.

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lation between ligand capture and Fab binding levels. For Biacore Flexchip, we devised a rapid assay that should prove to be particularly useful for GPCRs that are less stable than our CCR5 construct. Using either approach, we could bin the detergents into three groups: those that did not solubilize CCR5, those that solubilized but inactivated the receptor, and those that yielded active solubilized receptor.

Materials and methods

Materials

The Biacore 2000 and Flexchip optical biosensors, as well as instrument-specific consumables (e.g., sensor chips, affinity chip kits, amine-coupling kits, standard 96-well plates and covers), were obtained from GE Healthcare/Biacore (Uppsala, Sweden). Cf2Th canine thymocyte cells overexpressing the human chemokine receptor CCR5 tagged with a C-terminal linear peptide tag (C9: TETSQVAPA) were propagated by the National Cell Culture Center. Purified mouse anti-human CD195 (CCR5) monoclonal antibody 2D7 was provided by the National Institutes of Health AIDS Research and Reference Reagent Program and purchased from BD Biosciences (San Jose, CA, USA). The C9 tag-recognizing antibody 1D4 was purchased from the Technology Transfer Office at the University of British Columbia. Detergents were purchased from Anatrace (Maumee, OH, USA), DOPC/DOPS (7:3) lipid mixture was purchased from Avanti Polar Lipids (Alabaster, AL, USA), polyethylene glycol (PEG) 8000 was obtained from Promega (Madison, WI, USA), an ImmunoPure Fab Preparation Kit was obtained from Pierce (Rockford, IL, USA), and protease inhibitor tablets were purchased from Roche Diagnostics (Indianapolis, IN, USA). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Fisher Scientific (Rockford, IL, USA).

Preparation of 2D7 Fab

Here 1 ml of clone 2D7 monoclonal antibody (mAb) (cat. no. 555991, BD Pharmingen, 0.5 mg/ml in 50 mM Tris, 150 mM NaCl [pH 8.5], and 0.09% sodium azide, with conformational sensitivity as demonstrated by Navratilova et al. [1]) was dialyzed extensively against 20 mM sodium phosphate and 10 mM ethylenediaminetetraacetic acid (EDTA, pH 7.0) at 4 °C prior to Fab preparation. 2D7 Fab fragments were obtained and purified initially using the ImmunoPure Fab Preparation Kit (cat. no. 44885, Pierce). Using the Biacore 2000, fractions collected from the protein A column were tested for binding to 1D4-captured CCR5 solubilized as described in Ref. [7]. Fractions that bound CCR5 were passed over a sizing column to ensure purity of the 2D7 Fab. The activity of these postsizing column fractions was confirmed by binding to the CCR5 surface. Fractions containing 2D7 Fab were stored at 4 °C until use.

Immobilization of anti-C9 1D4 mAb on CM5 and Flexchip surfaces

Using Hepes-buffered saline (HBS: 10 mM Hepes and 150 mM NaCl [pH 7.4]) as the running buffer, 1D4 antibody (diluted in 10 mM sodium acetate [pH 5.5]) was immobilized at 25 °C via standard amine-coupling chemistry to densities of approximately 7000 resonance units (RU) on the four flow cell surfaces of a CM4 sensor chip. Using the same chemistry, 1D4 was immobilized across a Flexchip prototype dextran-coated affinity chip.

Preparation of detergent and lipid/cholesterol solutions

Stock solutions of detergents were prepared at 5% in water and stored at 4 °C until use. For solubilization trials, 20 μl of each detergent stock solution was dispensed into individual wells of a 96-well plate.

Dry films of DOPC/DOPS lipid were prepared as described by Navratilova and coworkers [7]. Immediately prior to receptor solubilization, 1 ml of solubilization buffer base (20 mM Tris, 0.1 M $(NH_4)_2SO_4$, 10% glycerol, 10% PEG 8000, and 0.07% cholesteryl hemisuccinate Tris salt [CHS] [pH 7.0]) was added to one aliquot of film, after which the solution underwent four vortex freeze–thaw cycles and was then diluted with chilled solubilization buffer (20 mM Tris, 0.1 M $(NH_4)_2SO_4$, 10% glycerol, 10% PEG 8000, and 0.07% CHS [pH 7.0] with one protease inhibitor tablet per 50 ml) to yield a lipid concentration of 0.33 mM.

Solubilization of C9-tagged CCR5

All steps were performed on ice or in a cold room. Approximately 6×10^6 CCR5-expressing cells were resuspended in 3 ml of the lipid-containing solubilization buffer, sonicated using a Soniprep 150 probe sonicator (six 1-s pulses), and diluted to 20 ml with lipid-containing solubilization buffer. Then 180 μl of this cell suspension was added to each well of a 96-well plate that contained 20 μl of detergent (see above). After sealing, the plate was spun gently on a vertical rotator for 6 h and then centrifuged. Supernatants were transferred to a new 96-well plate. As controls, each detergent screen included positive (buffer containing 0.33% Chaps/0.33% DDM [*n*-dodecyl-β-D-maltopyranoside]) and negative (no detergent added) controls.

CCR5 capture and 2D7 Fab binding using Biacore 2000

Receptor capture and Fab binding studies were performed at 25 °C using 50 mM Hepes, 150 mM NaCl, 0.02% CHS, 0.1% DDM, 0.1% Chaps, 50 nM DOPC/DOPS (7:3), and 0.2 mg/ml of bovine serum albumin (BSA) (pH 7.0) as the analysis buffer. In one binding cycle, three CCR5 preparations were captured on individual 1D4-coated flow cell surface, after which 2D7 Fab (100 nM) was injected for 4 min across the reference (1D4 alone) and three CCR5 surfaces. After a wash phase of 1 min, the surfaces were regenerated with 1% OGPS (*n*-octyl-β-D-glucopyranoside)/10 mM NaOH. The standard CCR5 solubilization condition (using Chaps/DDM) [7] was tested periodically throughout the screen to confirm receptor activity. Responses were processed and analyzed using Scrubber 2 (BioLogic Software, Campbell, Australia).

CCR5 capture and 2D7 Fab binding using Biacore Flexchip

The 1D4-coated chip was assembled into a continuous-flow microfluidic spotter from Wasatch Microfluidics (North Salt Lake City, UT, USA) [8,9]. We captured each of the 96 samples for 30 min on the chip. Once the chip was spotted, the flow cell was assembled and docked in the Flexchip instrument. Fab (100 nM) was flowed across the spots for 7 min, followed by buffer wash of 8 min. We used interspot referencing to correct for any instrument drift and then multiplied the responses by 15,000 to convert the signal from millidegrees (mdeg) to RU. Exported response data were processed and analyzed using Scrubber 2.

Results

GPCR detergent screening

Fig. 1 illustrates the basic steps we developed to screen solubilization conditions for GPCRs in a 96-well-plate format. Step 1 involved a brief sonication of approximately 6 million cells expressing C9-tagged CCR5 to create a homogeneous suspension.

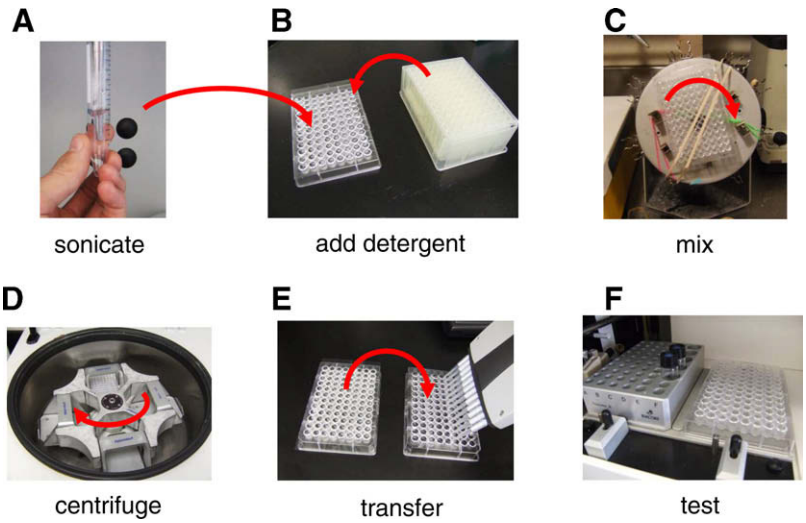

Fig. 1. Steps involved in screening detergents for GPCR solubilization: (A) sonicate cell suspension; (B) combine cell lysate with detergent panel; (C) mix to solubilize receptor; (D) centrifuge to remove cell debris; (E) transfer supernatant to sampling plate; (F) test for capture on tag-recognizing surface and for binding by conformationally sensitive analyte.

After sonication, the suspended cells were dispensed into a 96-well plate and different detergents were added to the individual wells. The plate was gently rotated for 6 h to promote receptor solubilization. After centrifugation to remove any precipitate, the supernatants were transferred and split into two new 96-well plates. One plate of solubilized samples was inserted into the autosampler of the Biacore 2000, and the second plate was used in the Flexchip analysis.

Screening detergents using Biacore 2000

With the serial processing Biacore 2000, we tested three detergent conditions within each assay cycle by injecting three differently solubilized CCR5 preparations across individual flow cell surfaces (Fig. 2). In this example, the first detergent effectively solubilized the receptor given that we see a significant increase in binding response during the capturing step (green sensorgram). The second detergent failed to solubilize CCR5 or completely blocks binding of the receptor onto the capturing surface (blue sensorgram). The third condition allows some capture of the receptor (pink sensorgram). After a 5-min washing step, the activity of the captured CCR5 was tested using a conformationally sensitive Fab injected at approximately 1200 s. From the binding responses, we see that, as expected, there was no binding of the Fab to the surface that did not capture any receptor (blue sensorgram). We see very little binding response on the third surface, which captured 300 RU of receptor, indicating that this condition might not stabilize the solubilized receptor (pink sensorgram). Finally, we see a significant binding response for the Fab to the receptor captured on the first surface (green sensorgram), indicating that this detergent condition maintains a folded conformation of the receptor at least in terms of Fab binding.

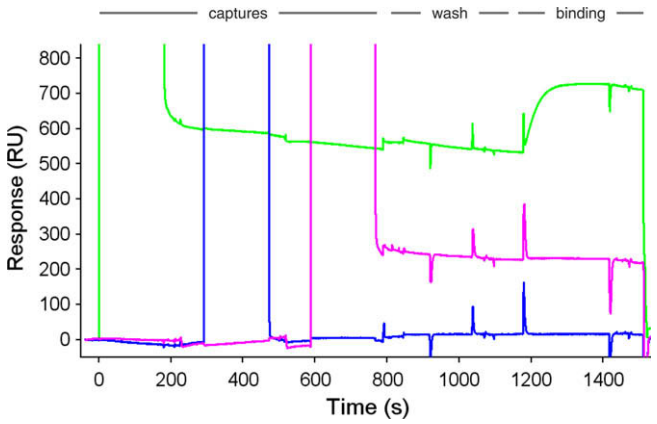

Fig. 2. One capture/binding cycle of screening detergents using Biacore 2000. CCR5 solubilized with one detergent was captured by immobilized 1D4 mAb in flow cell 2 (green), with another detergent in flow cell 3 (blue), and with a third detergent in flow cell 4 (pink). After a 300-s buffer wash, CCR5 capture levels were measured at $t = 1100$ s. At $t = 1180$ s, 2D7 Fab was injected across the captured receptor surfaces, as well as the reference surface (fc1, 1D4 mAb alone), for 4 min, after which 2D7 binding levels were measured and the 1D4 surfaces were regenerated. For the analysis of 96 detergents, this sequence was repeated 32 times. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Using the Biacore 2000, in one binding cycle we can quantitate how much receptor was captured and then compare these values with the amount of Fab binding. Another advantage of this capturing method is that the 1D4 surface can be regenerated. This allowed us to automatically screen a large panel of detergent conditions relatively efficiently. Including regeneration and washing steps, just under 24 h was required to analyze one 96-well plate of conditions.

Fig. 3 shows the overlaid binding cycles (separated by flow cell) from all 96 samples of this detergent screen. Many detergents did not solubilize the receptor, and several detergents produced solubilized but inactive CCR5. Only a small subset of detergents yielded active solubilized receptor.

The plot in Fig. 4 shows report points taken at the end of the wash step versus at the end of the Fab binding step. The conditions that failed to solubilize the receptor (blue and pink data points) cluster at zero. Those that solubilized but inactivated the receptor (orange data) are scattered along the x axis. Interestingly, many of the detergent conditions that yielded good binding signals for the Fab (green and red data) appear to sit on the dashed line. This linearity indicates that the activity of the receptor under these conditions is in fact the same; the Fab binding response intensities are different because they relate to the amount of receptor captured under each condition. Fig. 4 demonstrates the benefit of testing both receptor solubilization and activity levels.

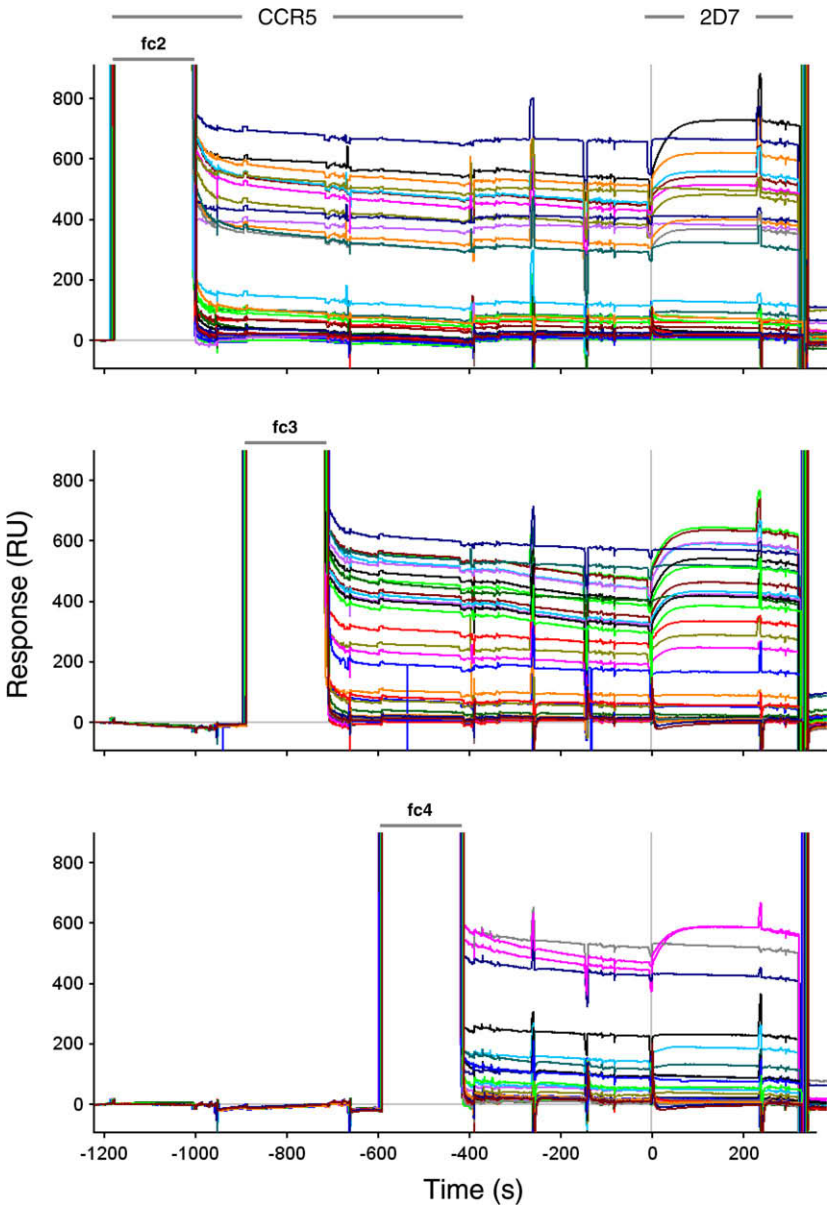

Fig. 3. Overlaid binding cycles, separated by flow cell, obtained for the panel of detergents using Biacore 2000.

Screening detergents using Biacore Flexchip

Using a Wasatch Microfluidics spotting instrument [8,9], we captured the solubilized CCR5 preparations at 96 positions on a 1D4 mAb-coated Flexchip slide. Fig. 5 shows an image of the slide's surface after receptor capture. The bright squares represent regions where receptor was deposited. Varying spot intensities represent varying levels of capture. Each ligand spot is well defined, indicating that none of the captured CCR5s spread to neighboring reference spots or contaminated nearby ligand spots.

Fig. 6 shows the responses for 2D7 collected across the spotted CCR5 array. In Fig. 6A, the response profiles from many of the spots show similar kinetics but different intensities. The 2D7 intensity level is a combination of the amount of CCR5 captured and its ability to bind the conformationally sensitive Fab. Fig. 6B shows report points taken at the end of the Fab injection plotted versus spot position. In this view, it is easier to see that a majority of the detergents did not support CCR5 binding activity, and this is in fact similar to the results obtained from the Biacore 2000 assay.

Correlation between Biacore 2000 and Flexchip screens

Fig. 7 compares the Fab binding levels measured using the Biacore 2000 and Flexchip assays. Maltosides with a C9 to C13 alkyl chain are shown in red, and maltosides with shorter and side chains are shown in pink. The green data points are for three nonmaltosides (Cymal-6, Cymal-7, and sucrose monodecanoate) that yielded active solubilized CCR5 (other nonmaltosides are shown in blue). Overall, the correlation between the two assays is very good, as indicated by the data points that align in a diagonal across the plot. Deviations from this diagonal are most likely due to differences in receptor capture levels. In both assays, the same 13 detergents were identified as viable solubilizing agents, all of which were easily discernible from the remaining detergents in the panel. Ten of the best detergents were maltosides. Also, in both assays the short and longest chain maltosides failed to support activity, whereas three nonmaltoside detergents maintained receptor activity. The similarity between these screening results further validates both sensor approaches.

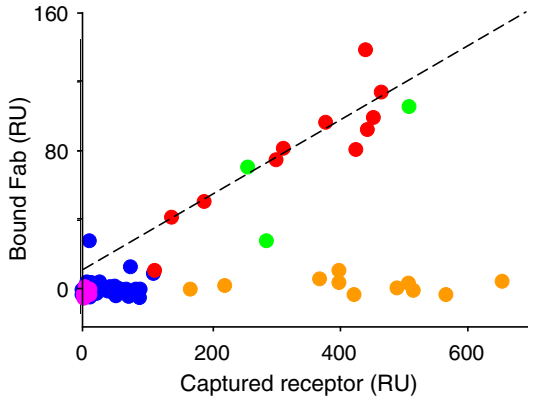

Fig. 4. Correlation between levels of captured CCR5 and 2D7 binding. Data points obtained for maltosides having an alkyl chain length of C9–C13 are shown in red, and pink depicts maltosides with an alkyl chain length of ⩽C8 or ⩾C14. Nonmaltoside detergents that poorly solubilized the receptor are shown in blue, orange depicts nonmaltosides that solubilized and inactivated the receptor, and green depicts nonmaltosides that yielded active solubilized receptor. The dashed line indicates a linear correlation between the capture and binding levels of the active solubilized CCR5 preparations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

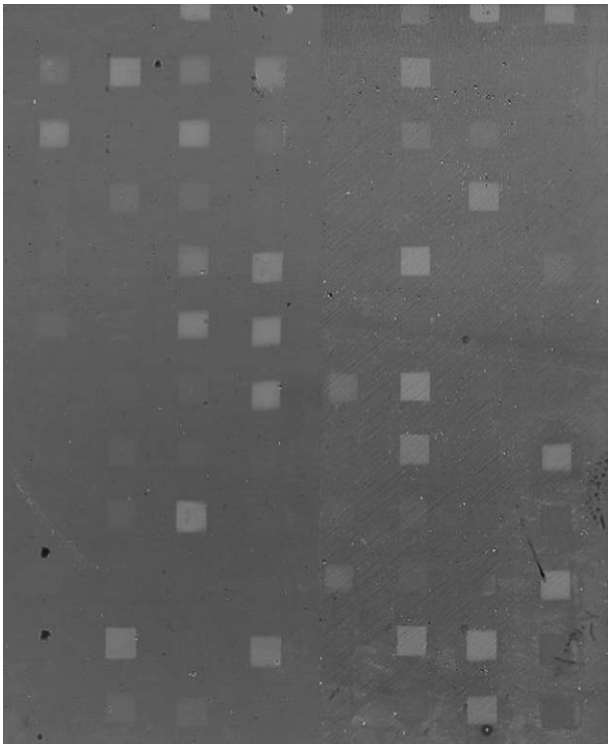

Fig. 5. Image from the Biacore Flexchip of a sensor surface patterned with receptor solubilized using different detergents. Using Wasatch Microfluidics' spotting technology, CCR5 (solubilized using different detergents) was deposited at discrete spots of the 1D4-coated chip surface.

Discussion

With recent breakthroughs in structural approaches to GPCRs [10–12], there is an increased interest in purification approaches for this class of receptor. The first challenge is to identify solubilization conditions that yield high amounts of receptor in a native conformation. Here we have demonstrated a method for testing large panels of conditions using the standard 96-well plate format. With this method, we identified detergents that effectively solubilized receptor while maintaining its activity. We can now test combinations of the most promising detergents to further optimize receptor solubilization and activity.

We also detailed screening approaches applicable for both serial and array biosensor platforms. The utility of serially processing biosensors is well established [13]. The benefits of these instruments are their ability to measure receptor capture levels and their sensitivity (the ability to detect small molecule binding is particularly important in GPCR studies). But the serial approach is time-sensitive. It requires more time to perform the analysis compared with the array approach, and because the analyses of different receptor preparations are staggered in time, we need to track receptor activity levels throughout the experiment. Also, this approach requires regenerating the capturing surface after each binding cycle. For this CCR5 system we could strip the C9-tagged receptor from the 1D4 surface, but we have examined other C9-tagged systems for which our regeneration conditions were not successful (data not shown). So, identifying appropriate regeneration conditions can be problematic or sometimes not possible (e.g., this approach cannot be used for biotin-tagged systems captured on standard streptavidin surfaces).

Our approach using the relatively new Flexchip array biosensor worked well for two major reasons. First, we used a continuous-flow spotting device in combination with prototype dextran-coated slides. Traditional pin-spotting methods for array biosensors in the past limited our choice of ligands to pure, highly concentrated solutions that could be dried without significant loss in activity (e.g., peptides, oligonucleotides, some antibodies) [9]. The continuous-flow spotter can extract the relatively fragile GPCR out of crude supernatant and maintain its microenvironment throughout the spotting process. In addition, we can perform the capturing step for as long as we like, making it possible to concentrate proteins from dilute solutions as well as crude solutions. The prototype dextran surfaces were essential to improving capture levels while at the same minimizing nonspecific interactions with the sensor surface. A limitation of the array, however, is that although we can estimate spot densities by looking at the intensity of each spot compared with nonspotted regions, this is not as simple or quantitative as in the serial platforms that monitor capture levels directly.

The array's second advantage is its speed. Ligand spotting and analyte binding can be completed in less than 1 h, and we can monitor binding at an array of ligand spots simultaneously. No regeneration of the surface is required, and all of the receptor preparations are solubilized and analyzed at the same time. The Flexchip's simultaneous analysis of all spots means that we do not need to account for loss of ligand activity over time. These features are particularly important for the analysis of unstable GPCRs. In addition, the array's rapid sampling allows flexibility in experimental design. With this approach, we can do time course studies and test combinations of solubilization conditions (e.g., various concentrations of different salts, lipids, and detergents as well as pH) in a large matrix. But with greater speed comes sacrifice. To date, array biosensors are limited in sensitivity to testing relatively large analytes.

It was important to see that both biosensor assays yielded the same results. The majority of the detergents that maintained CCR5 activity were maltosides with a C9 to C13 alkyl tail. Maltosides having a short (⩽C8) or long (⩾C14) alkyl chain were ineffective solubilizers. We identified three nonmaltosides (sucrose monodecanoate, Cymal-6, and Cymal-7 [shown in green in Fig. 7]) that also yielded good levels of solubilization and binding activity. The other nonmaltoside detergents did not solubilize the receptor and/or failed to maintain its activity.

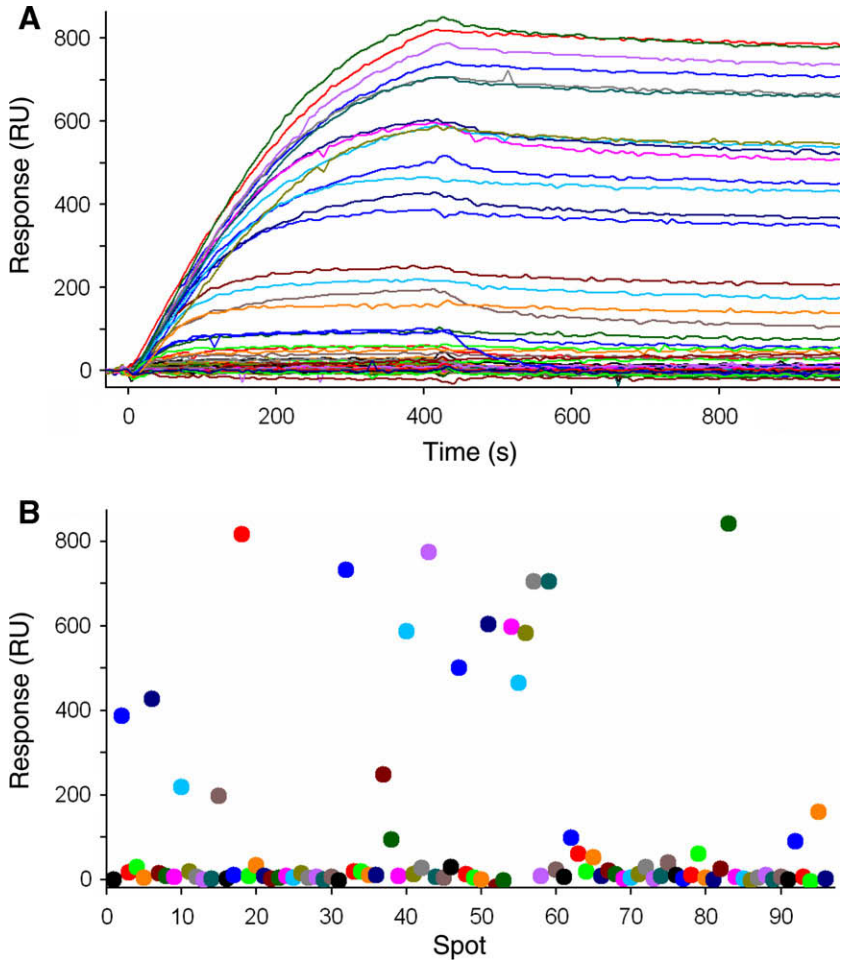

Fig. 6. 2D7 Fab binding to the matrix of CCR5 spots shown in Fig. 5. (A) Double-referenced responses obtained for 2D7 injected simultaneously across all of the different CCR5 preparations. (B) Response intensities at $t = 450$ s plotted against spot position.

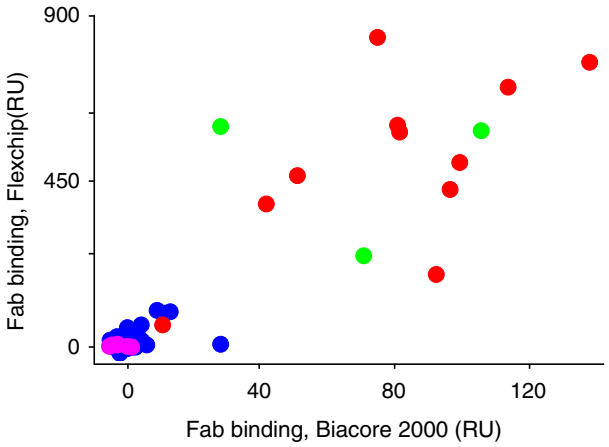

Fig. 7. Correlation between Fab binding levels measured using Biacore 2000 and Flexchip. Data points obtained for maltosides having an alkyl chain length of C9–C13 are shown in red, and pink depicts maltosides with an alkyl chain length of $\leqslant$C8 or $\geqslant$C14. Cymal-6, Cymal-7, and sucrose monodecanoate are shown in green, and the other nonmaltosides are shown in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

But the biggest disadvantage of the current serial and array biosensors is that both test analyte binding using only one buffer condition at a time. Although we solubilized the receptor under 96 different detergent conditions, the binding studies were performed with a single buffer. So, examining how components of the analysis buffer affect GPCR activity requires testing binding using one analysis buffer and then switching the instrument to another buffer and repeating the experiment. Although we identified promising solubilization conditions for CCR5 using established analysis buffer conditions, there may be a more optimal buffer system for this receptor. As array biosensor technology advances, we foresee systems that allow real-time capture and monitoring of activity under different analysis conditions in parallel.

Conclusions

The solubilization method outlined here provides the means to characterize 96 (or more) conditions in one experiment. Although we evaluated the viability of different detergents, this method can also be used to optimize other solubilization buffer components. Furthermore, we demonstrated that the serial and array biosensors both have distinct benefits and limitations for screening GPCR solubilization conditions.

Acknowledgments

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