Chapter 4

Label-Free Cell-Based Assay for Characterization of Biomolecules and Receptors

Diluka Peiris, Teodor Aastrup, Samuel Altun, Camilla Käck, Maria Gianneli, Davide Proverbio, and Lars M. Jørgensen

Abstract

We present a method to study the interaction between biomolecules and receptors present on the cell surface. This enables studies of molecular interactions in a natural biological context. As the analyte interacts with the receptors still intact on the cell surface, the experimental data provides complete dynamics and complexity of the interaction, thereby generating highly informative data. Attana's cell-based biosensor platform can be used to obtain this information from a diverse range of interactions as described in these protocols, which detail how to grow or capture cells on a surface, how to stabilize and visualize the cells on the surface, and how to set up assays to measure detailed interaction kinetics directly on the cell surface.

Key words Quartz crystal microbalance, Biosensor, Cell-based, Biomolecular interactions, Receptors, Affinity

1 Introduction

A key step in the drug development pipeline is the evaluation of affinity (equilibrium dissociation constant, K_D) of the new biotherapeutic molecule to its epitope. However, determination of kinetic parameters; association (k_a)- and dissociation (k_d) rate constants and affinity of a molecular interaction using purified ligand immobilized to a planar surface may not mimic the in vivo presentation of the ligand. Most methods used for studying cellular interactions (e.g., flow cytometry) require some form of labeling, usually fluorescent, to visualize the cells. This labeling can negatively influence the interaction by either conformationally changing the binding site, masking the binding site, or increasing the molecule's ability to bind undesired targets [1]. Cell surface ligands are found on the cell membrane in a milieu that includes diverse molecules in clusters, transmembrane proteins, lipids, GPI-anchored proteins, and carbohydrates—all of which might potentially interfere with an interaction between a biotherapeutic agent and its ligand.

Johan Rockberg and Johan Nilvebrant (eds.), *Epitope Mapping Protocols*, Methods in Molecular Biology, vol. 1785, https://doi.org/10.1007/978-1-4939-7841-0_4,

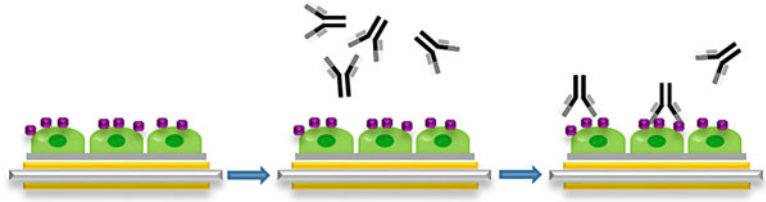

Fig. 1 Schematic diagram of cells on a surface with antibodies in flow over the surface and subsequent interaction event

To overcome the limitations of traditional biosensor platforms, a label-free cell-based biosensor assay platform has been developed and explored to obtain detailed kinetics and biological information on binding of biomolecules to intact cellular receptors [2, 3] (Fig. 1).

This approach has been used to study the interaction between humanized anti-Her2 antibody trastuzumab and ovarian carcinoma cells (SKOV-3) [4]. Further, in a recent study this cell-based biosensor platform has shown to be a valuable tool for evaluating changes in glycosylation and the effects these changes have on binding of chemotherapeutic agents in cancer [5]. The label-free cell-based assay detailed here can be used to study biomolecular interactions using adherent [2–4] or suspension cells [6], either fixed [7] or non-fixed [8]. Additionally, the biosensor can be used to analyze interactions directly on tissue specimens immobilized on the sensor surface [9].

This method starts with a choice between cell seeding (adherent cells) or cell capture (suspension cells). This is followed by an optional cell stabilization step and visualization of the sensor surface before inserting the surfaces in the instrument to perform the interaction studies.

2 Materials

1. PBS—Phosphate-buffered saline pH 7.4.
2. Water—All water used for the Attana Cell 200 Biosensor must be high quality water (18.2 MΩcm, 0.2 μm filtered).
3. Attana Cell 200 Biosensor.
4. Appropriate positive and negative control analytes.
5. Appropriate reference cell line.

2.1 Cell Seeding

2.1.1 Cell Growth and Visualization

1. COP-1 surface—Cell-optimized polystyrene surface. An Attana biosensor chip.
2. Culture media—any suitable cell culture media for the specific cell line.

3. Trypsin—EDTA solution.
4. Adherent cells—Any cell line that can grow in standard cell culture flasks can be grown on COP-1 surfaces.

2.2 Cell Capture

1. LNB-carboxyl surface—Low nonspecific binding carboxyl surface. An Attana biosensor chip.
2. Amine coupling kit—EDC (1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride), Sulfo-NHS (N-hydroxysulfosuccinimide), Ethanolamine (1 M, pH 8.5).
3. Immobilization buffer—e.g., 0.1 M sodium acetate pH 4.5.
4. Capturing molecule—*See* **Note 6**.
5. Suspension cells—Any primary cell or cell line that does not adhere to standard cell culture flasks. This method also works with adherent cells.

2.3 Cell Stabilization

1. Formaldehyde—3.7% methanol-free formaldehyde freshly prepared.

2.4 Surface Visualization

1. DAPI—4′,6-diamidino-2-phenylindole, 3 μM.
2. Fluorescent microscope.
3. Other staining—*See* **Note 10**.

2.5 Biosensor Assay

1. Analytes of interest.
2. 10 mM glycine pH 3.
3. 10 mM glycine pH 2.
4. 10 mM glycine pH 1.5.
5. 20 mM glycine pH 1.5.
6. 10 mM glycine pH 3 with 1 M NaCl.
7. 10 mM glycine pH 3 with 3 M NaCl.
8. 1 mM NaOH.
9. 10 mM NaOH.
10. 100 mM NaOH.
11. 1 mM NaOH with 3 M NaCl.
12. 10 mM NaOH with 3 M NaCl.
13. 100 mM NaOH with 3 M NaCl.
14. 0.005% SDS.
15. 0.01% SDS.

3 Methods

1. All solutions must be prepared in high quality water (18.2 MΩcm, 0.2 μm filtered).
2. When solutions are prepared by dissolving solids (e.g., powders or pellets), they must be filtered using 0.2 μm filters (*see* **Note 1**).
3. All pipetting should be done gently while avoiding contact between the pipette tip and the sensor surface.
4. The sensor surfaces must never be dry. Always leave a small amount of liquid, just enough to cover the surface, when changing buffers or performing washes.
5. Cover the surface whenever possible to prevent dust from settling on the surface.

3.1 Cell Seeding

1. Prepare the COP-1 sensor surfaces per the Instructions for Use.
2. Lift the cells from the cell culture flasks per standard protocols (*see* **Note 2**).
3. Dilute the cells to an appropriate cell density in culture media (*see* **Note 3**).
4. Add 700 μL of cell suspension to the COP-1 cultivation chamber.
5. Gently pipette the suspension up and down once to ensure an even distribution of cells.
6. Place the sensor chip under appropriate growth conditions to complete at least 1 cell cycle (*see* **Note 4**).

3.2 Cell Capturing

1. Dissolve or dilute the capture molecule in a suitable immobilization buffer (*see* **Note 5**).
2. Immobilize the capture molecule on an LNB-carboxyl surface (*see* **Note 6**).
3. Transfer the sensor surface (i.e., the gold chip) to a COP-1 holder.
4. Attach the cultivation chamber.
5. Prepare a cell suspension with a final density of 2×10^6 cells/mL in PBS (*see* **Note 7**).
6. Pipette 50 μL of the cell suspension on to the capturing surface making sure it covers the entire surface.
7. Incubate the cells for 30 min at room temperature.

3.3 Cell Stabilization

Cell stabilization is optional (*see* **Note 8**).

1. Remove the cell medium or buffer from the cultivation chamber.
2. Rinse the cells with PBS by gently pipetting up and down two times.
3. Wash the cells two times with PBS for 5 min each at room temperature.
4. Stabilize the cells with methanol-free formaldehyde 3.7% for 10 min at room temperature (*see* **Note 9**).
5. Rinse the cells with PBS by gently pipetting up and down two times.
6. Wash the cells two times with PBS for 5 min each at room temperature.
7. Add 700 μL PBS to the cultivation chamber. The surface can be stored at 4 °C for several days.

3.4 Surface Visualization

At this stage, it is possible to evaluate the surface coverage using a fluorescent microscope.

1. Incubate the cells with 3 μM DAPI for 5 min at room temperature (*see* **Note 10**).
2. Remove the DAPI from the cultivation chamber.
3. Rinse the cells three times with PBS by gently pipetting up and down.
4. Remove most of the liquid, leaving only enough to cover the surface.
5. Remove the cultivation chamber.
6. Visualize under a fluorescent microscope to evaluate the cell density (*see* **Note 11**) (Fig. 2).
7. Attach the measurement lid.
8. Using a pipette, gently fill the flow cell with running buffer by "inserting" the pipette tip into one of the two flow cell holes (Fig. 3) (*see* **Note 12**). The surface is now ready for use in the biosensor but can be stored at 4 °C for several days in a humidity chamber.

3.5 Biosensor Assay

The use of a dual channel system allows assessment and subtraction of the nonspecific binding to the sensor surface. A cell line that does not express the specific receptor can be used as the reference cell line. It is highly recommended to use a reference surface to analyze for nonspecific interactions to the sensor surface itself and cell membrane components other than the target (*see* **Note 13**). It is also recommended to use both positive and negative control sample injections before and after the analyte injection cycles (i.e., before data collection and after data collection, not between each cycle).

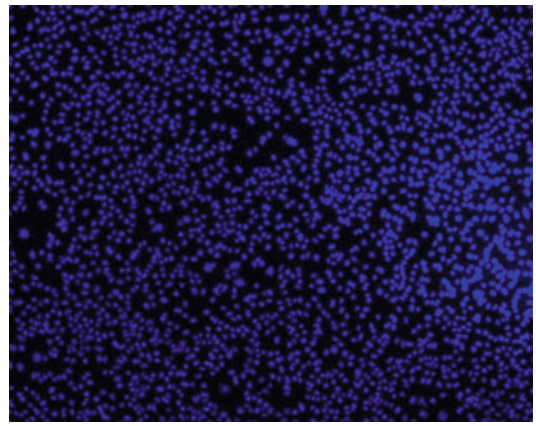

Fig. 2 Example of DAPI-stained cells grown on a COP-1 surface to ~80% surface coverage

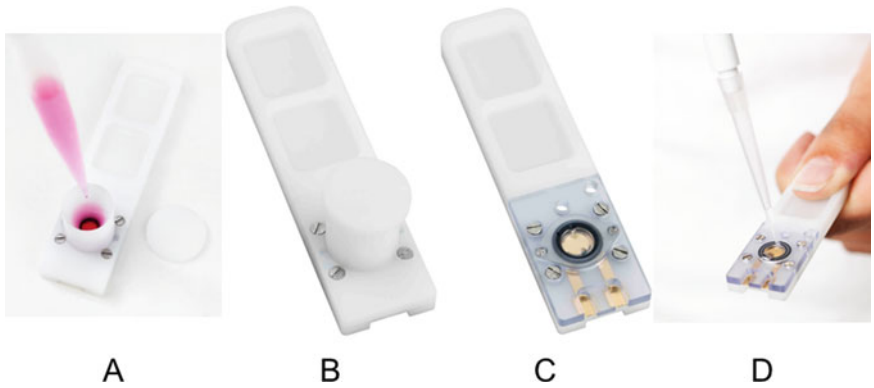

Fig. 3 COP-1 sensor surface: (**a**) with cultivation chamber, (**b**) with cultivation chamber with lid, (**c**) with measurement lid. (**d**) It shows the injection point for flushing aur bubbles out of the flow cell

1. Prime the instrument with a running buffer appropriate to the interaction (*see* **Note 14**).
2. Insert the sensor surfaces into the Attana biosensor.
3. Start the flow at 20 μL/min.
4. Set the temperature to the desired value.
5. Allow the baseline to stabilize (i.e., baseline drift of less than 0.2 Hz/min for a 10-min duration). This usually takes at least 4 h and can take up to 24 h.
6. Determine the analyte concentration range appropriate to the assay by injecting increasing concentrations of analyte starting with 0.1 K_D (if known) or 5 μg/mL (if unknown) and increasing by a factor 2 for each injection until no significant signal increase is detected. This gives the analyte concentration range

of the assay, which is usually close to the range of 0.1 K_D to 10 K_D.

7. Prepare regeneration solutions (*see* **Note 15**).
8. Inject the sample at half maximum concentration per **step 6**.
9. If prior knowledge exists, start with the most likely regeneration solution. Otherwise, start with the first solution on the list in **Note 15**.
10. Inject the regeneration solution in a short pulse (30 s for acidic solutions, 10 s for basic solutions or detergents). If part, but not all, of the analyte is removed (i.e., signal partially returns to the baseline) increase contact time (*see* **Note 16**). If none or only a very small amount of the analyte is removed, proceed to the next-regeneration solution on the list.
11. Inject the sample at half maximum concentration per **step 6**.
12. Regenerate the surface per **step 10**.
13. Repeat **steps 11** and **12** a total of five times. The last 3 cycles must show the same level of binding (*see* **Note 17**) (Fig. 4).
14. Determine the appropriate factorial dilutions to span the analyte concentration range per **step 6** while having five to seven different concentrations.

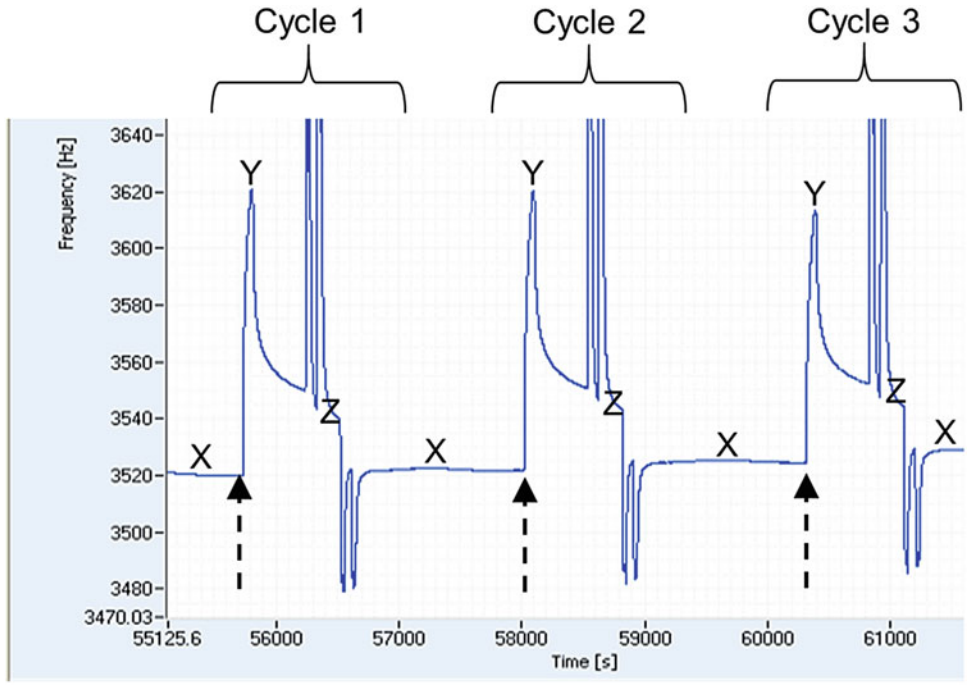

Fig. 4 Example of reproducible sample injections following regeneration. X denotes baseline, the arrows show the sample injection start, Y is the response peak, and Z is regeneration

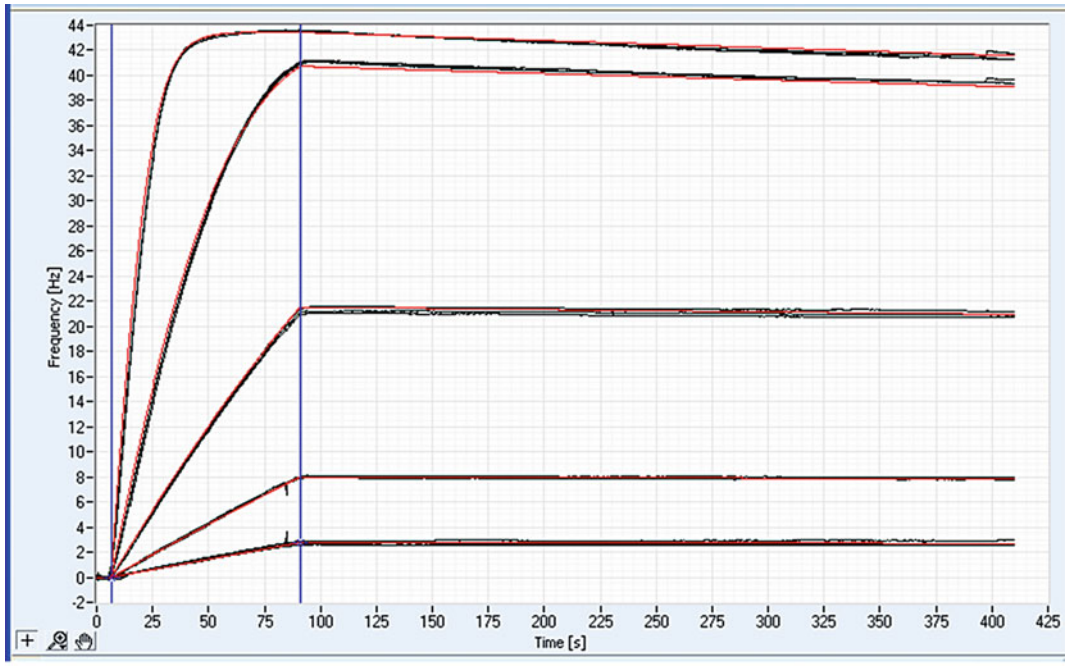

Fig. 5 An example sensorgram showing data (black lines) and curves fitted using Attester Evaluation (red lines)

15. Prepare the analyte dilution row per **step 14** by diluting the analyte in running buffer. Include the same number of blank injections (*see* **Note 18**).
16. Wash the biosensor loops with running buffer.
17. Inject a random blank with an association time of 105 s and a dissociation time of 300 s (or longer for strong binders).
18. Inject the corresponding sample as in **step 16**.
19. Regenerate per **step 6**.
20. Wash the biosensor loops with running buffer.
21. Repeat **steps 17–20** until all the samples are analyzed.
22. Data is now ready to be analyzed using Attester Evaluation (Attana) and/or TraceDrawer (Ridgeview) (Fig. 5).

4 Notes

1. It is not always possible to filter the solutions with a 0.2 μm filter. In that case, it can be acceptable to filter using a 0.45 μm filter keeping in mind that it increases the risk of clogging the instrument tubing.
2. It is recommended to passage the cells at least two times before using adherent cells in this protocol. Use standard methods

(e.g., trypsin-EDTA treatment) for lifting the cells from the cell culture flask.

3. A standard cell density is in the range of 60,000–1,20,000 cells/mL. Generally, a higher cell density is preferable as long as the cells are evenly distributed in the solution. The purpose of adjusting the cell density is to ensure cell coverage of 80% to achieve high sensitivity while minimizing the risk of multilayer formation.
4. The cells must be firmly attached and recovered from being lifted. This is usually achieved by allowing them to grow under standard conditions for the culture, e.g., in a CO_2 controlled environment at 37 °C and 5% CO_2. Cell coverage shouldn't exceed 80%.
5. A suitable immobilization buffer has a buffer capacity and pH approximately 1 unit lower than the pI of the molecule to be immobilized. Furthermore, it should not damage or cause irreversible conformational changes to the molecule to be immobilized.
6. Use the Instructions for Use of the Amine Coupling Kit to achieve a high density or saturated coverage of capture molecule on the LNB-carboxyl surface. This is achieved by multiple injections of the capture molecule until saturation is reached. A good capture molecule is any reasonably strong binder to a molecule present on the cell surface. Other criteria are that the capture molecule should be stable for the duration of the assays and not interact with the analytes. Common choices include antibodies and lectins.
7. It is possible to use a wide range of buffers for cell dilution as long as it does not contain components that inhibit the interaction between the capture molecule and its target.
8. There are advantages and disadvantages to using cell stabilization. Advantages include a more stable sensor surface allowing for longer assays and better signal-to-noise ratios. Disadvantages include the risk of altering the active site. To minimize the disadvantage, care must be taken to stabilize the surface as mildly as possible, as is done in this protocol [10].
9. Stabilizing with 3.7% formaldehyde for 10 min at room temperature is a good starting point. If a gentler stabilization is required, reduce the concentration of formaldehyde or perform the stabilization with cold formaldehyde either on ice or at 4 °C. Other cross-linking agents can also be used e.g. glutaraldehyde.
10. Any nucleic stain can be used. It is also possible to use surface staining, but it is recommended to remove the stain before the

assay or verify that the stain does not influence the interaction between the cell surface and the molecule of interest.

11. 80% surface coverage is a good target. Having less will significantly reduce the sensitivity of the assay.
12. When filling the flow cell, make sure that no air bubbles are trapped on the surface as this will greatly increase the baseline stabilization time. Air bubbles can be removed by gently flushing the flow cell with running buffer. If flushing does not remove the air bubbles, it may be necessary to remove and reattach the measurement lid.
13. It is a good idea to choose a reference surface that is as close as possible to the sample surface. The prioritized order of the reference surface is (1) the same cell line with a variant inactive target (e.g., a mutant), (2) the same cell line without the target molecule present (e.g., knockdown), (3) a different cell line not expressing the target, or iv) a surface without any cells on it.
14. The Attana biosensors can use a wide range of running buffers. It can either be simple buffers such as PBST or HBST, or more crude buffers containing protein contents such as serum or culture media. The running buffer can also contain blocking agents such as BSA or casein.
15. Surface regeneration is performed to remove remaining analyte before the subsequent analysis cycle can begin. Regeneration procedure must combine efficacy and preservation of the cells and their epitopes. Finding a regeneration solution that fulfils these criteria must be done empirically for every type of interaction pair. However, guidance can often be found in experiments using similar molecular species. To optimize the regeneration conditions, start with gentle regeneration solutions. If the analyte is not removed, a harsher solution is to be tried. The following regeneration conditions can be used as a guideline for cell sensor surfaces: 10 mM glycine pH 3–20 mM glycine pH 1.5, 10 mM glycine pH 3–20 mM glycine pH 1.5 with 1–3 M NaCl, 1 mM to 100 mM NaOH, 1 mM to 100 mM NaOH with 3 M NaCl, 0.005% to 0.01% SDS.
16. Increased contact time can be achieved by either increasing the length of the pulse in 20 s increments (up to 50 s) and/or repeated injections. Do not increase contact time beyond 100 s.
17. It is quite common to see an initial decrease in sensitivity during the first couple of cycles of sample—regeneration injection. The level of binding for the last three injections must be similar. If the difference in responses for the sequential injections decreases, but has not reached 0 yet, repeat Subheading 3.5, **steps 11** and **step 12** until the injections are sufficiently

stable or Subheading 3.5, **steps 11** and **step 12** have been repeated a total of ten times.

18. When preparing the individual blanks, it is best if they contain the same concentration of the solution used to store the analyte in as the corresponding sample injection. If this is not feasible, use running buffer as blanks.

References

1. Hawkins E, Cooper M, Campbell I (2006) Acoustic detection technology in the analysis of biomolecular interactions. Innov Pharm Technol 21:30–34
2. Peiris D, Markiv A, Curley GP, Dwek MV (2012) A novel approach to determining the affinity of protein–carbohydrate interactions employing adherent cancer cells grown on a biosensor surface. Biosens Bioelectron 35:160–166
3. Pei Z, Saint-Guirons J, Käck C, Ingemarsson B, Aastrup T (2012) Real time analysis of the carbohydrates on cell surfaces using a QCM biosensor: a lectin based approach. Biosens Bioelectron 35:200–205
4. Elmlund L, Käck C, Aastrup T, Nicholls IA (2015) Study of the interaction of trastuzumab and SKOV3 epithelial cancer cells using a quartz crystal microbalance sensor. Sensors 15:5884–5894
5. Peiris D, Spector AF, Lomax-Browne H, Ramesh B, Loizidou M, Welch H, Dwek MV (2017) Cellular glycosylation affects Herceptin binding and sensitivity of breast cancer cells to doxorubicin and growth factors. Sci Rep 7:43006. https://doi.org/10.1038/srep43006
6. Kalograiaki I, Euba B, Proverbio D, Campanero-Rhodes MA, Teodor Aastrup T, Junkal Garmendia J, Solís D (2016) Combined bacteria microarray and quartz crystal microbalance approach for exploring glycosignatures of nontypeable Haemophilus influenzae and recognition by host Lectins. Anal Chem 88 (11):5950–5957
7. Madani SY, Tan A, Dwek M, Seifalian AM (2012) Functionalization of single-walled carbon nanotubes and their binding to cancer cells. Int J Nanomedicine 7:905–914
8. Li X, Song S, Shuai Q, Pei Y, Aastrup T, Pei Y, Pei Z (2015) Real-time and label-free analysis of binding thermodynamics of carbohydrate-protein interactions on unfixed cancer cell surfaces using a QCM biosensor. Sci Rep 5:14066. https://doi.org/10.1038/srep14066
9. Clausen TM et al (2016) Real-time and label free determination of ligand binding-kinetics to primary cancer tissue specimens; a novel tool for the assessment of biomarker targeting. Sensing and Bio-Sensing Research 9:23–30
10. Tanaka KAK et al (2010) Membrane molecules mobile even after chemical fixation. Nat Methods 7(11):865–866