Measuring binding kinetics of ligands with tethered receptors by fluorescence polarization complemented with total internal reflection fluorescence microscopy

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Measuring Binding Kinetics of Ligands with Tethered Receptors by Fluorescence Polarization Complemented with Total Internal Reflection Fluorescence Microscopy

KWOK Ka Cheung

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Principal supervisor: Prof CHEUNG Nai Ho

Hong Kong Baptist University

July 2010
Declaration

I hereby declared that this thesis represents my own work which has been done after registration for the degree of PhD at Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or other institution for a degree, diploma or other qualification.

Signature: ____________________________

Date: July 2010
Abstract

The study of the binding between estrogen receptors (ER) and their ligands in vitro has long been of interest mainly because of its application in anti-estrogen drug discovery for breast cancer treatment as well as in the screening of environmental contaminants for endocrine disruptors. Binding strength was conventionally quantified in terms of equilibrium dissociation constant ($K_D$). Recently, emphasis is shifting towards kinetics rate constants, and off-rate ($k_{off}$) in particular. This thesis reported a novel method to measure such binding kinetics based on fluorescence polarization complemented with total internal reflection fluorescence (FP-TIRF). It used tethered receptors in a flow cell format. For the first time, the kinetics rate constants of the binding of full-length human recombinant ERα with its standard ligands were measured. $k_{off}$ was found to range from $1.3 \times 10^{-3}$ to $2.3 \times 10^{-3}$ s$^{-1}$. $k_{on}$ ranged from $0.3 \times 10^{5}$ to $11 \times 10^{5}$ M$^{-1}$ s$^{-1}$. The method could also be used to screen potential ligands. Motivated by recent findings that ginsenosides might be functional ligands of nuclear receptors, eleven ginsenosides were scanned for binding with ERα and peroxisome proliferator-activated receptor gamma (PPARγ). None of the ginsenosides showed significant binding to ERα, but Rb1 and 20(S)-Rg3 exhibited significant specific binding with PPARγ.
Acknowledgments

First of all, I wish to express my deep and sincere gratitude to my supervisor, Prof. N. H. Cheung, for his guidance throughout these years. I thank him for offering me the opportunity to do this interesting and attractive research, for teaching me to think like a physicist, and for providing me with continuous support and encouragement, especially during harsh times. I do not think that I can ever complete this work alone.

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<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>4OHT</td>
<td>4-hydroxy-tamoxifen</td>
</tr>
<tr>
<td>Abα</td>
<td>antibody targeting estrogen receptor alpha</td>
</tr>
<tr>
<td>AbR</td>
<td>anti-rabbit IgG</td>
</tr>
<tr>
<td>BF</td>
<td>bulk fluorescence</td>
</tr>
<tr>
<td>CoA</td>
<td>coactivator</td>
</tr>
<tr>
<td>DES</td>
<td>diethylstilbestrol</td>
</tr>
<tr>
<td>E2</td>
<td>estradiol</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>emCCD</td>
<td>electron-multiplied charge-coupled device</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>F</td>
<td>fluorescent labeled ligand</td>
</tr>
<tr>
<td>f</td>
<td>bound fraction</td>
</tr>
<tr>
<td>FA (or A)</td>
<td>fluorescence anisotropy</td>
</tr>
<tr>
<td>FP</td>
<td>fluorescence polarization</td>
</tr>
<tr>
<td>FP-TIRF</td>
<td>fluorescence polarization with total internal reflection fluorescence</td>
</tr>
<tr>
<td>GEN</td>
<td>genistein</td>
</tr>
<tr>
<td>H12</td>
<td>helix 12</td>
</tr>
<tr>
<td>HCB</td>
<td>humidity-controlled box</td>
</tr>
<tr>
<td>hERα-FP</td>
<td>full length human ERα with fluorescence polarization assays</td>
</tr>
<tr>
<td>IC50</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>KD</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>k_{off}</td>
<td>off-rate</td>
</tr>
<tr>
<td>k_{on}</td>
<td>on-rate</td>
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<tr>
<td>L</td>
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<tr>
<td>LBD</td>
<td>ligand binding domain</td>
</tr>
<tr>
<td>NHS</td>
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<td>phosphate buffered saline</td>
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<td>PLL</td>
<td>poly-L-lysine</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
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<td>PPD</td>
<td>protopanaxadiol</td>
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<td>PPT</td>
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<td>raloxifene</td>
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<td>RBA</td>
<td>relative binding affinity</td>
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<tr>
<td>SERMs</td>
<td>selective estrogen receptor modulators</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TIRF</td>
<td>total internal reflection fluorescence</td>
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CHAPTER 1  INTRODUCTION

Nuclear receptors are ligand-inducible transcription factors which regulate the development and the maintenance of homeostasis of the organism. An example is the estrogen receptor (ER). It is an important member of the nuclear receptor super-family which binds with female hormones to trigger downstream effects and eventual proliferation of mammary cells. ER has two isotypes: ERα and ERβ. ERα has been shown to be linked to breast cancer development. Selective estrogen receptor modulators (SERMs) are therefore developed to compete with endogenous hormones for the same ERα receptor site but will not cause cell proliferation or other harmful side-effects [1]. Another health issue related to nuclear receptors is environmental endocrine disruptors. They are hormone-like substances found in the environment that disrupt the normal endocrine functions of the body to cause potential health problems, such as abnormal fetal developments [2]. High-throughput screening of environmental contaminants for such disruptors is therefore necessary. Meanwhile, the mechanism of ER-mediated intra-cellular signaling through ligand binding is still poorly understood [3, 4, 5]. For all these reasons, the binding of ER with its ligands is being actively investigated.

For the purpose of anti-estrogen drug discovery and for high-throughout screening of endocrine disruptors, receptor-ligand bindings are studied in vitro for better control of parameter values and to avoid in vivo complications [6, 7]. To quantify binding affinity, the dissociation constant $K_D$ of the receptor-ligand complex is conventionally measured [8]. Recently, however, attention is shifted to the kinetic rate constants, the off-rate $k_{off}$...
in particular, for drug characterization [9]. It is because live cells, being open systems, are not in equilibrium and steady-state constants measured in test tubes may not be relevant. In contrast, $k_{off}$ of unimolecular dissociation events remains unchanged be it in test tubes or live cells.

Accordingly, an assay to measure the kinetics rate constants of receptor-ligand interaction \textit{in vitro} is sought after. The method should be sensitive, accurate, reliable, reproducible, and convenient. It has to be fast for screening of potential ligands. Although the sure-fire method is still to be developed, three general classes of techniques are potential candidates. They are assays based on (1) radiolabels, (2) mass differences, and (3) fluorescence. Radio-assays made use of the competitive displacement of radiolabeled ($^3$H or $^{131}$I) ligand by other ligands for the same receptor. Although they offer sensitive detections, they are handicapped by limited shelf life, hazardous handling of radioactive materials and problematic waste disposal [10, 11, 12]. Mass-difference assays, such as surface plasmon resonance (SPR), are widely used [13, 14]. Fluorescence assays, especially those based on fluorescence polarization (FP) anisotropy and evanescent wave excitation are also gaining acceptance.

The prime advantage of mass-difference assay is label-free, so target ligands can be measured as they are. The challenge is to measure small ligands such as estrogens when the added mass (mass difference) is minute. In order to boost the weak signal, higher probe density is required, but at the expense of local reagent depletion. On the other hand, fluorescence assays offer single-molecule detection sensitivity but require dye-labeling of the analytes. We will show that the best of both worlds is possible, that sensitive fluorescence techniques can be developed to measure receptor-ligand binding
kinetics of label-free analyte ligands. The method is based on monitoring the fluorescence anisotropy of a fluorescent reporter ligand as it competes with a dark ligand. We will show that it is best to have the probe receptors tethered on a substrate, in a flow-cell format. This way, only very minute amounts of reagents are needed, reagents can be changed at any stage of the binding reaction, and micro-array type parallel processing is possible. And because receptors are immobilized on the substrate surface, the reporter ligand can be excited by evanescent waves in the total internal reflection fluorescence (TIRF) configuration. The TIRF signal can be used to check the FP signal for consistency.

Using our FP-TIRF setup, we have succeeded in measuring the kinetics rate constants, both on and off –rates, of a series of standard ligands with ERα. We have also demonstrated the fast screening of numerous ginsenosides for binding with ERα and peroxisome proliferator-activated receptor gamma (PPARγ). The steroid-like structure of ginsenosides and their documented potency in disease treatment motivated our screening study.

The organization of the thesis is as follows. The motivation and objectives of this work are described in the present chapter. Chapter 2 reviews receptor-ligand interactions and some of the more common measurement techniques. Chapter 3 gives the experimental details, from instrumentation to data run protocols. Chapter 4 covers the experimental results and their interpretations. The measurement of the kinetics rate constants of fluorescent and dark ligands are described in detail, together with the results of the fast screening of ginsenosides for binding with nuclear receptors. Chapter 5 summarizes the key results and highlights the significance of this work.
CHAPTER 2  BACKGROUND

This chapter reviews the basic concepts of receptor-ligand interaction and describes how that interaction is measured conventionally.

2.1  Receptor ligand interaction

In this section, the conventional wisdom and the biological significance of receptor ligand interaction were reviewed, under the following headings, (1) nuclear receptor superfamily, (2) estrogen receptor structure and function, (3) breast cancer and drug discovery, (4) endocrine disruptors and contaminant screening, (5) peroxisome proliferator-activated receptors, and (6) ginsenosides as potential ligands.

2.1.1  Nuclear receptor superfamily

Nuclear receptors (NR) are a superfamily of ligand-inducible transcription factors which interact with specific DNA sequence and other proteins to modulate specific gene expression in order to maintain the metabolism, homeostasis and development of organisms. Over 100 nuclear receptors are identified, and they are classified into different classes depending on their function and sequence homology [15]. All nuclear receptors contain the activation function 1 (AF-1), DNA binding domain (DBD), hinge region, ligand binding domain (LBD) containing activation function 2 (AF-2) and the remaining C-terminus. AF-1 is a ligand-independent binding region which interacts
weakly with the other regions but synergizes with AF-2 binding. DBD contains two zinc fingers which bind with specific DNA sequence called hormone response elements (HRE). LBD is the most important part of the receptor. The receptor dimerizes and modulates the recruitment of cofactors upon ligand binding.

2.1.2 Estrogen receptor structure and function

Estrogen receptor (ER) binds specifically to female hormone or other estrogen ligands and then triggers downstream effects leading to the proliferation of mammary cells. ER, together with estrogen-related receptors (ERR), androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), all belong to the steroid hormone receptor class in the nuclear receptor superfamily. ER has two isotypes, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). They share ~97% sequence homology in DBD and ~55% sequence homology in LBD, as shown in Figure 2.1. That is why they bind to the same DNA sequence called estrogen response element (ERE), and bind equally strong to the female hormone estradiol (E2), and share most of the specific ligands as agonists and antagonists, shown in Figure 2.3 [16].

The secondary and tertiary structures of ERα are shown in Figure 2.2. Upon specific ligand binding to the hydrophobic binding pocket of the ER-LBD, if the ligand is an agonist, the LBD folds to a conformation when the helix 12 (H12) caps the ligand binding pocket and exposes the nearby hydrophobic groove for the further binding of the LxxLL motifs of a coactivator (CoA) which is needed for transcription, as shown in
Figure 2.4. On the other hand, if the ligand is an antagonist, the H12 flips outward to a position that blocks the binding of the LxxLL motifs of CoA [16], also shown in Figure 2.4. There are studies showing that the ligand binding can also modulate the dimerization interaction of two LBDs of an ER dimer. The ER dimer can either be a homodimer (ERα-ERα or ERβ-ERβ), or heterodimer (ERα-ERβ) [17]. There were studies showing that ligand binding at LBD of ER affected its DBD function [18]. These allosteric and cooperative effects of ER binding are still under study.

![Diagram of ERα and ERβ primary structures](image.png)

**Figure 2.1** The primary structures of ERα and ERβ, showing their homology and functions of different regions. [19]
Figure 2.2  (A) The primary structure of ERα.  (B) The secondary structure of ERα-LBD.  (C) The tertiary structure of ERα-LBD. [16]

Figure 2.3  The top three chemicals are agonists of ER. The bottom three chemicals are antagonists of ER.
Figure 2.4  The conformation of ERα-LBD upon ligand binding. Left: The coactivator docking pocket (light green) formed by helices 3, 5 and 12 upon agonist binding. Middle: Docked coactivator (purple) with LxxLL motif. Right: Upon antagonist binding, helix 12 swings further to block the coactivator docking pocket. [16]

The classical model of ER signaling for transcription is that the estradiol (E2) goes into the cytoplasm and binds to ER monomer. The activated ERs form homodimers or heterodimers and then bind to the DNA sequence ERE and further recruit other coregulators needed for transcription [20], as shown in Figure 2.5. However, there are studies showing that ligand-independent and ERE-independent pathways can also initialize transcription, in which phosphorylated ERs bind to ERE, or activated ERs bind to other DNA sequences by association of other transcription factors, as shown in Figure 2.6, plus there are nongenomic effects resulting from ER ligand binding as well [20].
The complete picture of ER signaling for transcription is still unclear. For example, is ER mainly in the form of dimer or monomer in the cytoplasm? Can an ER dimer go from the cytoplasm into the nucleus? Does ER bind to the ERE as a monomer or dimer initially? Does ER bind the CoA first or the ERE first? The quantitative characterizations of allosteric and cooperative effects of ER interactions are also lacking.

**Figure 2.5** The classical model of ER signaling for transcription. Estrogen bound ERα form homodimer and hence binds to estrogen response element ERE and coactivator GRIP-1 to initiate transcription. [21]
Figure 2.6  Ligand-independent ER pathways and nongenomic pathways. (1) The classical model of ER signaling for transcription. (2) ligand-independent transcription activation through phosphorylation of ER. (3) ERE-independent transcription activation through association of transcription factors. (4) Nongenomic pathways. [20]

2.1.3  Breast cancer and drug discovery

Due to radiation, chemicals, virus, heredity or DNA replication errors, the mutation of DNA occurs in a cell. If this DNA-damaged cell does not repair itself nor commit suicide (apoptosis), it becomes a cancer cell. The cancer cell will then undergo cell divisions and have more and more DNA mutations to disrupt normal cell growth balance. Finally, the cancer cells undergo uncontrolled growth and gradually form larger cancerous tissue, called tumor. This cancer tissue gradually invade the surrounding
normal tissues to disrupt normal function of the organ, and may also invade the blood vessels to transport the cancer cells to other parts of the body and grow at new locations [1].

According to the World Health Organization (WHO), cancer will replace heart disease to become the top human killer by 2010. Twenty-seven million new cancer cases are to be expected by 2030 [22, 23]. Cancer prevention and treatment is certainly of top priority. Breast cancer is one of the most common cancers. It was shown that for breast cancer, ERα was highly expressed in cancer cells to regulate their proliferation through female hormone induced transcriptions. Meanwhile, ERβ was found to be expressed in normal cells. Therefore, a major breast cancer therapy is the administering of an anti-estrogen drug which acts as an antagonist of ERα and competes with the female hormone to block the transcription process and prevent proliferation of breast cancer cells, as shown in Figure 2.7. However, these anti-estrogen drugs also affect the signaling in normal cells and cause undesirable side effects in the body. For example, the most common drug tamoxifen also activates uterine receptor to increase the risk of uterine cancer development. Therefore, selective estrogen receptor modulators (SERMs) are being developed which are designed to suppress breast cancer cell development and at the same time have no side effect, or even have beneficial effect to the body [24, 25]. Until now, there is no perfect SERM for breast cancer therapy [24]. SERM chase is still on, and the development of newer techniques to aid drug discovery and to enhance the understanding of receptor-ligand binding is keenly sought after.
2.1.4 **Endocrine disruptors and contaminant screening**

Another important health issue related to ER ligand interaction is the environmental hazard caused by so-called endocrine disruptors. Endocrine disruptors are hormone-like chemicals that exist in the environment around us. For example, they may be found in drinking water, consumer products, food additives, chemical wastes, etc. Once up taken, they may engender hormonal effects through ER and AR signaling in the human body to cause long-term public health problems. Detection of this kind of environmental hazard is very challenging. Very large scale screening of potential endocrine disruptors is needed but the technology does not yet exist. The National Toxicology Program (NTP) of the National Institute of Environmental Health Sciences (NIEHS) in the US reviewed current *in vitro* ER and AR ligand binding assays [2] and concluded that no standardized binding assay can be made from current methods due to the lack of validation data. The development of a simple, sensitive, reliable, robust, and high-throughput technique to screen receptor-ligand binding is urgently required.

*Figure 2.7*  Action of anti-estrogen drug for breast cancer therapy.
2.1.5 Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPARs) belong to a group of the nuclear receptor superfamily [26]. PPARs have three isotypes, PPARα, PPARβ and PPARγ. Like other nuclear receptors, PPARs have AF-1, DBD, hinge region and LBD. However, PPARs only form heterodimers with the retinoid X receptors (RXRs) and no homodimers [26]. The tertiary structures of PPARγ(LBD) and the PPARγ-RXR heterodimer are shown in Figure 2.8. PPARs are lipid inducible transcription factor which regulate the gene expression for lipid and glucose metabolism [27]. Cholesterols, phospholipids and fatty acids are major lipids in human body. PPARγ is found to play a key role in adipogenesis (generation of fat cells) and is associated with atherosclerosis, obesity, insulin resistance diabetes (high blood glucose level) and cancer [27]. For diabetes treatment, insulin-sensitizing drugs, e.g. thiazolidinediones in Figure 2.9, were developed as PPARγ agonists to reduce insulin resistance and to improve insulin sensitivity in order to regulate normal blood glucose level in the body [26, 27].

Figure 2.8 Left: Ligand-binding domain (LBD) of the PPARγ. Right: LBDs of the
PPARγ-RXR heterodimer.

Figure 2.9 Left: thiazolidinedione. Right: three members of thiazolidinedione derivatives.

2.1.6 Ginsenosides

For over thousands of years, panax ginseng C.A. Meyer is one of the most popular herbs used in traditional Chinese medicine. Recently, it plays an increasingly important pharmacological role in western medicine as well. Ginsenosides are the small molecules of active ingredients in ginseng. More than thirty types of ginsenosides are classified into two main types: protopanaxadiol (PPD) and protopanaxatriol (PPT). They differ by the various sugar moieties attached at the different positions as shown in Figure 2.10. The pharmacological effects of ginsenosides include the modulation of angiogenesis and their effects on the central nervous system. They also have specific wound-healing and anti-tumor effects [28]. As can be seen from Figure 2.10, the
structures of ginsenosides are similar to steroids (Figure 2.3). It was suggested that their pharmacological effects might arise from interaction with nuclear receptors such as ERs. For that reason, their screening for binding with nuclear receptors will help clarify the issue.

Figure 2.10  Left: Panax ginseng.  Right: The chemical structures of ginsenosides.
2.2  *In-vitro* binding assays

As mentioned in Chapter 1, there are three main types of techniques for the measurement of *in vitro* receptor ligand binding. They are radio-assays, mass difference assays and fluorescence assays. Although radio-assays offer sensitive detection, they are hampered by limited shelf life, radioactive hazards and waste disposal problems [10, 11, 12]. In this section, we will give an overview of mass difference assays and fluorescence assays. We will explain their pros and cons, and outline how we can build on them to develop a better alternative method.

2.2.1  Mass difference assays

Mass difference assays are widely used to measure binding kinetics between targets in solution and probes immobilized on surfaces. The surface mass increase upon target binding to tethered probes is monitored. The most common mass difference assay is surface plasmon resonance (SPR). These assays have the advantage of measuring label-free ligands, but the sensitivity can be low if the mass increment is small.

2.2.2  Surface plasmon resonance (SPR)

Surface plasmon is the electromagnetic wave which propagates along the interface between a metal and a dielectric. It is created by a light beam incident at the surface. The property of this electromagnetic wave is very sensitive to the refractive index change
of the interface caused by mass or thickness increases. As a result, the intensity of the reflected light beam also changes, and this change is usually measured [13]. The SPR biosensor measures the mass change due to the association and dissociation of molecular complex tethered on the metallic surface in order to obtain the kinetics rates. This is commonly used for biological binding kinetics studies, such as antibody-antigen, DNA-protein, and receptor-ligand interactions. The advantage of SPR biosensor is label-free analytes [14]. However, the sensitivity can be low when the molecular weight of the analytes is low. The limit of detection is about 10 pg mm\(^{-2}\), which is equivalent to one hundred bovine serum albumin (\ (~ 66 kDa) bound to an area of 1 \(\mu m^2\). For estrogen receptor binding studies using SPR, large molecular weight analytes are preferred. For example, ER dimerizing with tethered ER monomer [29], ER binding to tethered ERE [30], and ER binding to tethered hormone [31] were investigated. For the case of small ligands as analytes, the mass increment will be minuscule and the surface density of the tethered probes will have to be increased in order to boost the binding mass. But then, the reagents will be depleted or accumulated near the surface and the interpretation of the kinetics data will be very complicated [32]. This probably explains why small ligands as analytes could only be studied by SPR experts [33]. Moreover, for bindings involving multiple partners, if only mass but no other identifier is measured, the many binding events cannot be easily decoupled.

A less common mass difference assay is the quartz crystal microbalance with dissipation (QCM-D). Similar to SPR, it measures the mass change due to binding but can also measure the structural change at the same time [30], and therefore has similar
advantages and limitations as SPR. The limit of detection of QCM-D is also about 10 pg mm\(^{-2}\).

**Figure 2.11** The typical setup of SPR with flow channel, measuring the angle shift of the maximum reflectance upon binding mass increases.

### 2.2.3 Fluorescence assays

Fluorescence assays are another commonly used technique to measure binding affinity and kinetics. It is based on the detection of a fluorescent reporter ligand. The fluorophores may be a dye chemically bonded to the biomolecule of interest, such as proteins, DNAs, lipids and small ligands. Unlike mass difference assays, dye labeling is generally required. Fluorescence measurement can be very sensitive, and even single fluorophores can be detected. Moreover, specific binding signal from a particular fluorophore can be spectrally or temporally selected so that multi-partner interactions can
be de-scrambled. In the following sections, several common fluorescence assays that are used to study binding kinetics will be discussed [34].

2.2.4 **Total internal reflection fluorescence (TIRF)**

Total internal reflection fluorescence (TIRF) makes use of the thin (~ 100 nm) evanescent wave produced by a total internally reflected laser beam at the glass-water or quartz-water interface to excite the surface immobilized fluorophore [35]. The emissions are either imaged by a charge-coupled device (CCD) or detected by a photomultiplier tube (PMT). The time course of the emission intensity gives the binding kinetics of the dye-labeled ligand with the surface-tethered probes, usually in a flow cell configuration. The sensitivity of TIRF is extremely high and can reach single molecules detection level [36, 37]. Vogel used TIRF to study the binding kinetics of serotonin 5HT$_3$ receptor with a novel fluorescent ligand. They obtained the on and off-rates of the fluorescent ligand. Because of its relatively small off rate, they could only measure the dissociation constants of nonfluorescent competitor ligands but not their on and off-rates [38].
2.2.5 Total internal reflection with fluorescence correlation spectroscopy (TIR-FCS)

Total internal reflection with fluorescence correlation spectroscopy (TIR-FCS) is built around a microscope. It is another fluorescence technique for exciting/viewing a very small analyte volume. The z dimension is again restricted by evanescent wave of TIR. The x-y dimension is restricted by a μm-size pin-hole at the image plane of the microscope. When the fluorescent ligands enter or exit the detection volume to bind with or dissociate from surface receptors, the fluorescence intensity will change. The time-dependent auto-correlation function will yield the off-rate of the fluorescent ligand. The advantages of TIR-FCS are that it can also determine the receptor coverage and the z-diffusion constant of the fluorescent ligand. However, because of the small detection volume, the signal is generally weak even for high receptor coverage, and the study of nonfluorescent ligands will be difficult. Thompson demonstrated interesting
applications of this technique, and anticipated the extension to nonfluorescent ligands. So far, only kinetics of fluorescent ligands were measured [39, 40].

Figure 2.13 The setup of TIR-FCS, measuring the fluorescence correlation of fluorescent molecules diffuse in and out of the viewed volume during the binding process [39].

2.2.6 Total internal reflection with fluorescence photobleaching recovery (TIR-FPR)

Total internal reflection with fluorescence photobleaching recovery (TIR-FPR) is based on the photobleaching of surface bound fluorophores at time zero, and the monitoring of their gradual displacement by bright fluorophores originally in solution [41]. The advantages of TIR-FRP are that the off-rates of the fluorescent ligand can be measured directly and no sample flow is needed. However, this method cannot
determine the kinetics of nonfluorescent ligands because they will be confused for bleached fluorophores.

2.2.7 **Total internal reflection with reflectance interferometry (TIR-RIf)**

Total internal reflection with reflectance interferometry (TIR-RIf) is a technique that uses TIRF to measure the fluorescence signal of surface fluorophores and RI of to measure the change in normal reflectance of the substrate surface [42]. Akin to SPR, the normal reflectance changes upon ligand binding to surface tethered receptors. Again, no dye labeling is necessary and RI can give the absolute mass added to the surface once the instrument is calibrated. However, it is limited by the same low sensitivity of ~ 10 pg mm⁻². If the ligand is fluorescent, the TIR part can detect the signal very sensitively.

![Diagram](image)

**Figure 2.14** The setup of TIR-RIf, measuring both the fluorescence from TIR and the change in fluorescence of normal reflectance upon ligand binding [42].
2.2.8 **Fluorescence polarization (FP)**

Fluorescence polarization (FP) is a powerful technique for measuring the interaction of small fluorescent ligands with large receptors. The small (~ $10^2$ D) fluorophore in solution is first excited by a linearly polarized laser beam. The parallel and perpendicularly polarized components of the fluorescence emissions are measured. The polarization anisotropy is computed [43]. If the fluorescent ligand is unbound, within its fluorescence lifetime, it already tumbles enough cycles in solution to scramble the parallel and perpendicular components and the polarization anisotropy is small. If it is bound to a large ($10^4$ D) receptor, its rotation is slowed and the polarization anisotropy is high. FP is a simple and robust technique and can be used to measure the binding of dark ligands through their competition with a bright reporter ligand. It has been widely used in numerous biological and pharmacological studies [44, 45, 46] to measure the binding affinity (in terms of the dissociation constant $K_D$ or $IC_{50}$) of various receptors with their respective ligands. As for measuring kinetics rate constants, FP has only been used to study fluorescent but not dark ligands [43].
Figure 2.15  Upper: Typical FP setup for measuring the polarization of emission from small fluorescent molecules. Lower: The degree of polarization distinguishes the fast tumbling free fluorescent molecule from the larger complexed ones. [47]

2.2.9 Free probes versus tethered probes

*In vitro* receptor-ligand binding measurements are carried out either in bulk solution
with stopped-flow mixing or in flow cells with tethered probes. Free probes in solution have the advantages of having probes in their native forms and the setup is generally simpler. On the other hand, flow cells with tethered probes use much less chemicals and allow reagent changes at any stage of the reaction. This format also allows parallel processing as in microarrays and therefore is scalable for high throughput assays [48, 49].
CHAPTER 3 METHODS

We aimed at measuring the binding kinetics of ERα and its ligands, as well as the screening of ginsenosides for binding with nuclear receptors. We combined two techniques, fluorescence polarization (FP) and total internal reflection fluorescence (TIRF), to do the measurements; and we used kinetics modeling to analyze our data. In this chapter, we will first describe the experimental setup and the sample preparation procedures. The flow of a typical data run will be explained next. The way binding data were processed and analyzed will be covered in the end.

3.1 FP-TIRF setup

A previous student K.M. Yeung had built a TIRF apparatus [50]. We modified it to include the FP function, as shown in Figure 3.1(a). The entire setup was built around an inverted microscope (Olympus IX-71). The flow cell was made of a quartz slide and a cover slip, and sealed with high vacuum grease. The fabrication of the flow cell will be described later. The flow cell was clamped onto the traveling stage of the microscope and coupled with immersion oil (n = 1.47, Electron Microscopy Sciences, 16916-01) to an isosceles Brewster prism (CVI Melles Griot, IB-10.5-68.7-SS) fixed relative to the microscope stand, so that the flow cell could freely translate without moving the prism. To implement FP and TIRF at the same time, 473-nm diode-pump solid-state (DPSS) laser (Photop DPBL-9050) was split into two balanced beams, and incident on the same prism at two different angles. One laser beam was incident at 70°,
which was larger than the critical angle of the quartz-water interface (=65°), so an evanescent wave extended about 100 nm into the solution of the sample cell. The other laser beam was steered with mirrors to incident at 41°, and penetrated through the flow cell. The two incident laser beams were S-polarized in order to produce the desired excitation of the fluorophores in the flow cell [43]. The laser power of the two beams was balanced with a neutral density filter. The bulk fluorescence (BF) and TIR fluorescence (TIRF) were collected by a 10× microscope objective (NA = 0.25), filtered by a long-pass filter (Chroma HQ485lp) and directed through a broadband polarizing cube beamsplitter (Newport PB 3) for separation of the parallel (∥) and perpendicular (⊥) components of BF and TIRF. Finally, the BF∥, TIRF∥, BF⊥ and TIRF⊥ were aligned and imaged onto an electron-multiplied CCD (emCCD, Andor iXon-em+ 897). The typical image captured was shown in Figure 3.1(b).

3.2 Setup optimization and calibration

Before data capture, the FP-TIR setup was adjusted and calibrated. The emCCD was first set to equilibrate at -80°C, 300 electron multiplier gain, 8 × 8 binning and 0.2 second exposure per frame, in order to optimize the detection sensitivity. For maximum signal-to-noise ratio (S/N) at an acceptable photobleaching rate, the power density of the BF and TIR laser spots at the sample cell were controlled and balanced by inserting neutral density filters. Laser spot sizes were adjusted by translating the converging lens. The laser (traveling wave) power densities of the BF and TIR spots were 0.38 and 0.14 W cm⁻², respectively. The BF and TIRF excitation beams were steered for proximal (~ 0.5
mm along the 35 mm channel) but non-overlapping images on the emCCD. This ensured near identical probed areas of the two beams for meaningful comparison of BF and TIRF signal.

It is generally impossible to perfectly balance the // and \( \perp \) output beams. Instead, their slight imbalance can be corrected by scaling the \( \perp \) signal by a so-called \( G \)-factor [43]. The \( G \)-factor for our setup was found by using the Beacon 2000 One-Step FP Standardization Kit (Panvera P2581). The reference buffer (1 nM fluorescein, FITC, solution) was injected into the sample cell and the // and \( \perp \) signals of both the TIRF and BF spots were measured on the emCCD. The \( G \)-factor was found to be 0.95. All the fluorescence anisotropy data were corrected with this \( G \)-factor. Using the same data, we also calibrated the BF spot brightness against the concentration of FITC. Because the fluorophore of the fluorescent ligand was also FITC [44], this allowed us to determine the concentration of the reporter ligand from the measured BF signal intensity.
Figure 3.1  (a) The FP-TIRF setup.  (b) A typical image captured by emCCD showing four spots.  From top to bottom: BF//, TIRF //, BF⊥ and TIRF⊥.

3.3  Flow cell fabrication and fluidics

In this section, we will describe (1) the fabrication of the flow cell, and (2) the fluid flow control.
### 3.3.1 Flow cell fabrication

75 mm × 25 mm × 1 mm quartz slides were purchased from Technical Glass Product Inc.. 40 mm × 22 mm × 0.13~0.16 mm cover slips were purchased from Menzel-Glaser. Six 2-mm holes were drilled on each new quartz slide as shown in Figure 3.2. Scratching of the slide surface had to be carefully avoided while drilling. The quartz slides and cover slips were sonicated in acetone (Tedia AS-1112) for 30 minutes, followed by sonicing twice in Milli-Q water (18.2 MΩ·cm) for 5 minutes. For recycled slides, the acetone cleaning step was repeated until all high vacuum grease was removed. The quartz slides and cover slips were then sonicated in 0.5 M potassium hydroxide, KOH (Sigma-Aldrich 221473) in 50% ethanol, etOH (Merck) for 60 minutes at 60°C, followed by sonicating twice in Milli-Q water for 5 minutes. This KOH-etOH cleaning step was repeated twice to ensure complete cleaning. The clean quartz slides and cover slips were either stored in Milli-Q water, or used immediately for sample cell fabrication.

To fabricate a sample cell, clean quartz slides were dried in 99% nitrogen. Clean cover slips were sonicated in ethanol, followed by drying in an oven at 150°C for 2 minutes. High vacuum grease (Dow Corning) was patterned on a clean cover slip with a needleless syringe as applicator. The cover slip was then laid on a clean quartz slide, grease side down, to form channels as shown in Figure 3.2. The channels were then filled with phosphate-buffered saline, PBS (Gibco 21600-051) that contained 1.47 mM potassium phosphate monobasic (KH₂PO₄), 8.1 mM sodium phosphate dibasic (Na₂HPO₄), 2.67 mM potassium chloride (KCl), 137.93 mM sodium chloride (NaCl),
and 0.02% sodium azide NaN₃. The filled cell was placed in a humidity-controlled box (HCB) at room temperature (RT), ready for the surface coating step. The thickness of the channel was about 30 µm, as measured with a microscope. The typical dimension of each channel was 35 mm × 5 mm × 0.03 mm.

3.3.2 Fluidics

To inject reagents, about 15 µL of the solution was pipetted into the inlet well of the flow channel. The solution was sucked in when the original contents in the channel was drawn out by dipping lint free paper at the channel outlet. For the inflow of the fluorescent ligand F, the increase in the bulk fluorescence signal indicated the influx speed. It was found that the complete replacement of the channel contents took just a few seconds. By monitoring the BF intensity, the actual concentration of F in the flow channel could be tracked accurately over time.

![Diagram of flow cell](image)

**Figure 3.2** Top: Top view of flow cell. Bottom: Side view of flow cell. The direction of solution flow is indicated by dashed arrows.
3.4 Channel surface preparation

In the binding experiments, we sometimes wanted to have the receptors and ligands freely moving in the solution, and adsorbing minimally on the substrate (quartz slide and glass cover slip) surfaces. At other times, we purposely tethered the receptors on the substrate surfaces. In this section, we will explain how that can be realized by the various coatings.

3.4.1 Coating with PEG

It is known that polyethylene glycol (PEG) hardly interacts with proteins and most ligands [51]. If coated on a surface, it will serve as an excellent passivating layer. We sometimes coated the quartz slides with PEG to block the nonspecific adsorption of receptors and ligands. The coating procedure is as follows. Poly-L-lysine, PLL (Sigma P8920) was diluted to 0.05% in PBS. 15 μL of this solution was injected twice into a new channel, and incubated overnight at RT, followed by flushing three times with 30 μL PBS. PEG labeled with N-hydroxysuccinimide, mPEG-NHS (Nektar 2M4K0D01) was dissolved in PBS at 5 mg/ml, injected into the channel and incubated for 2 hours at RT, followed by flushing three times with 30 μL PBS. The sample cell was stored in the HCB at RT until use [52, 53].
3.4.2 Immobilization of primary and secondary antibody

In most other experiments, we purposely tethered ERα on the slide and cover slip surfaces. The tether comprised of several links, as shown in Figure 3.3. The procedure for immobilizing each link, up to the anti-ERα antibody, is described below. PLL was diluted to 0.05% in PBS. 15 μL of this solution was injected twice into a new channel, and incubated at RT for 30 minutes, followed by flushing three times with 30 μL PBS. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDC (Sigma 39391) and N-hydroxysuccinimide, NHS (Aldrich 130672) were dissolved in ≥99.9% DMSO (Sigma D8418) at 60 mg/mL and 450 mg/mL respectively, and stored at -20°C. Repeated freeze-thaw cycle and long-term storage was avoided. Goat anti-rabbit IgG, AbR (Zymed 81-6100 or Zymed 65-6100) was diluted to 0.25 mg/mL in PBS with 1.5 mg/mL EDC and 11 mg/mL NHS. 15 μL of this solution was injected into the channel, and incubated at 4°C for 2 hours, followed by flushing three times with 30 μL tris-buffered saline, TBS (24.7 mM Tris, 2.8 mM KCl, 139 mM NaCl), and incubated for at least 30 minutes in order to passivate the remaining reactive groups. Anti-ERα, clone 60C (Upstate 05-820) was diluted to 0.02 mg/mL in PBS. 15 μL of this solution was injected into the channel, and incubated at 4°C for at least 30 minutes, followed by flushing three times with 30 μL PBS. The sample cell was stored in the HCB at 4°C until the next step of capturing ERα.
3.4.3 Receptor preparation and tethering

Stock solution of human recombinant estrogen receptor α, ERα at 2 μM (Invitrogen Panvera, P2187) was divided into aliquots of 30 μL each and stored at – 80°C for long-term storage, or -20°C for short-term usage. Repeated freeze-thaw cycle should be avoided per vendor's suggestion. The binding buffer (100 mM potassium phosphate, pH 7.4, 0.25% to 1% DMSO, 0.1 mg/mL bovine gamma globulin BGG and 0.02% sodium azide NaN₃) was used in all ERα–ligand binding experiments. The concentration of DMSO in the binding buffer was kept at or below 1% per vendor's suggestion. To tether the receptor, the binding buffer was preloaded in an antibodies-coated channel. An aliquot of ERα was thawed and diluted to 40 nM in binding buffer on ice. 15 μL ERα in binding buffer was injected into the channel, and incubated for 15 minutes at RT to allow the capturing of ERα by the anti-ERα on the surface, followed by three flushes with blank binding buffer. The channel with tethered ERα, as shown in Figure 3.3, was now ready for ligand binding experiments.
Figure 3.3  The immobilization scheme for receptor ligand binding study.
3.5 Ligand preparation

Solution of the green fluorescent ER ligand F (Fluormone™ ES2, Invitrogen Panvera, P2645) at 400 nM in methanol was first diluted to 200 nM in 50% methanol and 50% DMSO as stock solution. This solvent combination avoided methanol volatility and DMSO freezing. The stock solution was divided into aliquots of 20 μL each and stored at –20°C until use. Estradiol (E2), diethylstilbestrol (DES), tamoxifen (TAM), 4-hydroxytamoxifen (4OHT), raloxifene (RAL) and genistein (GEN) were all purchased from Sigma (product E8875, D4628, T5648, H7904, R1402 and G6649, respectively). Ginsenosides were purchased from Fleton. The purity of all the above ligands from Sigma and Fleton were ≥98%. All ligands were dissolved in DMSO at 1 mM as stock solution and small aliquots were stored at -20°C until use. For binding experiments, ligand stocks were diluted serially in DMSO until the final 100-fold when it was diluted in the binding buffer. This ensured complete dissolution of ligands. The final ligand concentration could therefore be deduced reliably based on dilution ratios.

3.6 Program for real-time data capture and display

A kinetics run usually consisted of fast (seconds) and slow (minutes) events. Slow events were recorded by one second long movies of 5 Hz frame rate taken at one minute intervals. If spatial averaging was needed, the slide would be translated and ten or more such movie segments would be taken in prompt succession; the process would be repeated every minute. To capture fast events, such as the influx of Fluormone F and
their binding with and dissociation from receptors, 30-second movies of one spot were taken; the slight photobleaching was gauged by comparing with neighboring non-irradiated spots. Movies were saved as raw TIF files and were processed by a MATLAB program in real time. The program calculated the average brightness over user-defined areas such as the four spots shown in Figure 3.1 (b) as well as peripheral areas that served as background. The net signal was plotted against frame number, as illustrated in Figure 3.4. As can be seen from the figure, the TIRF and FP traces were displayed as the reaction progressed. At the end of a data run, the data traces in text format and the raw TIF file were stored for off-line processing and analysis.

![Figure 3.4](image)

**Figure 3.4** Left: Matlab program for real-time data capture and display. Red and pink curves are the respective BF // and BF⊥ signal. Blue and cyan curves are the TIRF // and TIRF⊥, respectively. Green dots are the FA calculated from BF // and BF⊥. Right: The areas of interests (AOIs) for averaging BF //, BF⊥, TIRF // and TIRF⊥, together with the background measurement.
3.7 Data runs

Three different kinds of binding experiments were performed. They were (1) binding kinetics measurements with surface tethered receptors and free ligands, (2) binding kinetics measurements with free receptors and ligands, and (3) fast screening of ginsenosides for binding with tethered receptors. Each will be described below.

3.7.1 Kinetics data capture for surface tethered receptors

The FP-TIR setup was optimized and calibrated. The ligand binding experiment was performed in the dark. While the experiment could be divided into sessions, these sessions were performed in succession using the same channel. In the first session, the aim was to generate enough kinetics data to model the ERα-F binding kinetics. 10 µL F at 1 nM in binding buffer was flowed into the ERα-tethered channel. Flow stopped within 3 seconds. Meanwhile, movies were taken for 30 s at 5 Hz frame rate. The // and ⊥ components of TIR and BF were computed and displayed at 0.2 s time resolution. After the first 30 s, 1-s movies were taken every minute out to 5 minutes. The total BF intensity, total TIRF intensity, bulk fluorescence anisotropy and TIF fluorescence anisotropy were computed in real-time and displayed. Typically, ERα-F binding equilibrated within 1 to 2 minutes. The photobleaching rate of F was determined by continuous recording of the same illuminated spot. 50 frames of a non-irradiated neighboring area were taken for comparison. The above procedures were then repeated for two more cycles in order to generate a few more equilibrated states for kinetics
modeling of ERα-F (see next chapter). In the first cycle, 15 µL of blank binding buffer instead of F was injected. In the second cycle, 10 µL F at 1 nM in binding buffer was injected.

In the second session, the aim was to generate data to model ERα-L binding kinetics where L was a dark ligand. 15 µL of freshly prepared L at known concentration in binding buffer was flowed into the same channel, while movies were taken for 30 s, followed by periodic 1-s movie segments every minute until the competition between F and L for ERα reached steady-state. That occurred typically after 10 to 15 minutes.

In the third and final session, the aim was to displace all F from ERα in order to measure the minimum FP value (corresponding to 100% free F). The value was needed for the data analysis and had to be determined for each sample channel because it depended somewhat on the surface chemistry and cleanness and the condition of the binding buffer. To measure it, a mixture of 20 µM estradiol (E2) and 0.5 nM F in binding buffer was injected into the channel. After 5 minutes, movies were taken. The minimum FP anisotropy was typically about 80 mA. This complete displacement of F by an excess of E2 also served as a positive control.

3.7.2 Kinetics data capture for free receptors and ligands in solution

The protocol for bulk reaction kinetics measurement was again performed in successive sessions. In the first session, the aim was to measure the steady-state binding of ERα-F. 7.5 µL of ERα at 10 nM and 7.5 µL of F at 2 nM in binding buffer were mixed at the inlet port at time zero. Within 5 s, the mixture was flowed into a
PEG-coated sample channel. Inflow was completed within 15 s. 1-s movie segments were taken every min out to 5 min when steady-state was reached. The bound fraction of F was about 0.9 at steady-state. Extent of photobleaching was determined by continuous recording of the same illuminated spot. The image was compared against non-irradiated neighboring areas.

In the second session, the aim was to measure the ERα-F dissociation kinetics. 15 µL of blank binding buffer was added to the outlet port. This pushed the channel contents (the ERα-F solution) back to the inlet port. The original ERα-F solution was sucked into a pipette that was preloaded with 1 µL of E2 at 100 µM. The mixture was injected into the sample channel again. This step was completed within 15 seconds. 1-s movie segments were taken at 30 seconds and every minute out of 5 minutes. The low TIR signal showed that the nonspecific adsorption of F on the surface was negligible. At the same time, the FP anisotropy was high at the end of the first session. That indicated that nearly all the ERα–F binding occurred in solution.

3.7.3 Screening ginsenosides for binding with nuclear receptors

The screening of ginsenosides for ERα binding was performed in nearly the same manner as surface binding kinetics measurement. The preparation step was to first get about half or more of the tethered ERα complexed with F at steady state. This was achieved by flowing 10 µL of F at 1 nM in binding buffer into the ERα-tethered channel. The system reached equilibrium in less than 5 min.

15 µL of a freshly prepared ginsenoside at their maximum soluble concentration
of 10–50 μM in binding buffer was flowed into the sample channel. 1-s movies were taken every min until 5 min or until the F-ginsenoside competition equilibrated.

If no ginsenoside binding was observed, a good fraction of tethered ERα was still complexed with F and no regeneration of ERα-F was necessary. A second ginsenoside could be flowed in right away.

If ginsenoside binding occurred, the ERα-F complex had to be regenerated. It could be done in two ways. The first method is to inject blank binding buffer, allow the bound ginsenoside to dissociate, and inject blank buffer a second time to remove as much ginsenoside as possible. F was then flowed in again to produce the ERα-F surface. The second method, which is probably better, is to displace the bound ginsenoside by a high concentration of a weakly binding ligand such as bisphenol A, followed by flushing with blank buffer and then F solution.

To screen ginsenosides for binding with PPARγ, GST-tagged PPARγ-LBD (Invitrogen PV4545) was tethered by anti-GST antibody (Zymed 71-7500) which in turn was captured by anti Rabbit IgG. Two green fluorescent PPARγ ligands (Panvera PV3356 and Invitrogen PV4896) were used as reporters. GW1929 (sigma G5668) and GW9662 (sigma M6191) are used as PPARγ standard ligands with purity ≥98%. The binding buffer was available as part of a commercial kit (Panvera PV3355). All the above ligands were prepared for screening according to the protocols in section 3.5.

3.8 Data analysis – From fluorescence anisotropy to bound fraction

Our kinetics analysis was based on measuring the fluorescence anisotropy of F.
The anisotropy parameter $A$ is defined by [43],

$$A = \frac{I_{//} - I_{\perp}}{I_{\text{total}}},$$

(3.1)

where $I_{//}$ and $I_{\perp}$ are the bulk fluorescence intensities of $F$ with polarization parallel and perpendicular to the incident polarization, respectively; and $I_{\text{total}}$ is the total fluorescence intensity. Values of $A$ is usually reported in mA, where 1 mA = $10^{-3}$.

If the dipole $\mu$ of $F$ is oriented randomly, $I_{\text{total}}$ can be shown to be [43],

$$I_{\text{total}} = I_{//} + 2I_{\perp},$$

(3.2)

and $A$ can be converted to the bound fraction $f$ of fluorophores [43],

$$f = \frac{A - \underline{A}}{\overline{A} - A}.$$  

(3.3)

In the last equation, $\underline{A}$ is the minimum $A$ value measured off 100% free $F$ in solution and $\overline{A}$ is the maximum $A$ value measured off 100% bound $F$. We generated 100% free $F$ by two independent methods: (1) by mixing $F$ and receptors with a huge excess of a competitor ligand and (2) by flowing $F$ into a sample channel coated with PEG. We produced 100% bound $F$ again by two independent means. The first was by allowing $F$ to bind to tethered ER$\alpha$, waited till equilibrium, and then promptly flushed out all free $F$ and measured $A$ at that instant. The second was based on mixing $F$ with excess ER$\alpha$ in solution in a PEG-coated channel. All results were found to be consistent and the value of $\underline{A}$ and $\overline{A}$ was about 80 and 320 mA, respectively.

We will show in the next chapter that even for tethered ER$\alpha$, $\mu$ of bound $F$ was oriented randomly. We could therefore make use of Eq. (3.1) through Eq. (3.3) to determine $f(t)$ from measured $A(t)$. 

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3.9 Kinetics modeling

We determined the rate constants by simulating \( f(t) \) with the following kinetics model. When we flow \( F \) onto tethered ER\( \alpha \), the time rate of change of the surface density of the receptor-\( F \) complex \( [RF] \) is given by [8],

\[
\frac{d[RF]}{dt} = k_{on}[F][R]_{total} - [RF] - k_{off} [RF],
\]

(3.4)

where \([F]\) is the concentration of free \( F \) in solution, \([R]_{total}\) is the surface density of apo and complexed receptors, and \(k_{on}\) and \(k_{off}\) are the respective on and off-rate constants. Expressing Eq. (3.4) in terms of the bound fraction \( f \), we have,

\[
\frac{df}{dt} = k_{on}(1 - f)(\rho_{total} - f[F]_{total}) - k_{off} f,
\]

(3.5)

where \(\rho_{total}\) is the equivalent volume concentration if all the tethered receptors are dissolved in solution, and \([F]_{total}\) is the equivalent volume concentration of free and complexed \( F \).

Two features about Eq. (3.5) should be pointed out. First, \([F]_{total}\) could be dynamic but it was measurable because it was proportional to \(I_{total}\). Once \(I_{total}\) was calibrated, the time course of \([F]_{total}\) would be known. Second, all the dynamical variables in Eq. (3.5) were assumed to be position independent. This is the so-called rapid mixing assumption [32]. Its justification will be given later.

By solving Eq. (3.5) numerically to best fit the measured \( f(t) \), we could determine the three unknowns, \(\rho_{total}\) and the two rate constants.

When a dark ligand \( L \) competes with \( F \) for the same receptor, Eq. (3.5) needs to be modified to become two coupled differential equations,
\[ \dot{f} = k_{on} (1 - f) \rho_{total} - f [F]_{total} - f \tilde{f} [L]_{total} - k_{off} f, \quad (3.6) \]

and

\[ \dot{f} = \tilde{k}_{on} (1 - \tilde{f}) \tilde{\rho}_{total} - f [F]_{total} - \tilde{f} [L]_{total} - \tilde{k}_{off} \tilde{f}, \quad (3.7) \]

where variables with tildes are the L analogs of F. In contrast to \([F]_{total}, [L]_{total}\) could not be measured in real time based on fluorescence. Instead, it was determined from dilution ratios based on known concentration stocks. Two precautions were therefore necessary. First, the solubility of L in the dilution buffer became a critical concern. This explained our use of DMSO in the early dilution steps. Second, \([L]_{total}\) was assumed to be time independent. Its variation during reagent injections could not be accounted for and simulations of those transient moments had to be avoided. With these issues properly addressed, we could determine the five unknowns in Eq. (3.6) and (3.7), \(\rho_{total}\) and the four rate constants, by solving the coupled differential equations numerically to best fit the measured \(f(t)\).

### 3.10 Rapid mixing assumption

The concept of rapid mixing could be explained by the following example. Consider a channel with bound and free F in dynamic equilibrium and no fluid flow. Quite obviously, the concentration of F in solution, \(C\) is a constant independent of time and space. Now imagine injecting a solution of comparable \([F]\) plus excess competitors L. After a few seconds, the fluid returns to static. But the bound F coming off (dissociation time constant about 30 s, see next section) will be replaced by L. So \(C\)
becomes a time-dependent $C(t)$. The dissociated F will diffuse away from the surface. If this diffusive mixing is too slow, the free F may accumulate near the surface, resulting in an $C(t,z)$ where $z$ is the distance from the surface.

To determine if diffusive mixing is rapid enough or not, we can consider the number of F molecules dissociated from unit area of tethered receptors in time $\tau$, where $\tau$ is the time for a free F to diffuse half the height $h$ of the sample channel. We use half height because there are tethered receptors on the quartz slide as well as on the cover slip; drifting to the middle will mean thorough mixing. $\tau$ is given by $\tau = \frac{h^2}{8D}$ where $D$ is the diffusion constant of free F.

These freed F molecules are released in a volume of (channel height $h$)$\times$(unit area). The concentration of released F, relative to the background [F], is therefore given by,

$$\frac{[F]_{\text{released}}}{[F]_{\text{bkgd}}} = \frac{k_{\text{off}} [R]_{\text{total}} h}{8D [F]_{\text{bkgd}}}.$$  \hspace{1cm} (3.8)

If the ratio is much less than 1, mixing is rapid enough.

For association events, we can derive in a similar fashion the criteria for rapid mixing. We can also derive the criteria more rigorously by requiring that the F flux at the surface to be equal to the association flux minus the dissociation flux, and normalizing this flux conservation relation to a dimensionless form. The result is identical to Eq. (3.8), within a factor of 2. We will show in the next chapter that rapid mixing was realized and justified in our experiments.
Our target was to establish a sensitive, accurate, reliable, reproducible and convenient technique (1) to measure the kinetics rate constants of receptor-ligand interaction, and (2) to screen ginsenosides for binding with nuclear receptors. Specifically, for target (1), we focused on estrogen receptor alpha ERα and its binding with standard label-free ligands. For target (2), we focused on two nuclear receptors, ERα and PPARγ.

After reviewing existing techniques (Chapter 2), we concluded that measurements using FP-TIRF in a flow cell configuration with surface immobilized receptors would be the most suitable. Our reasons for choosing fluorescence assays were, (1) fluorescence assays were the most sensitive and fast; (2) the local concentration of the reporter ligand [F] could be measured in real time; and (3) dark ligands could still be analyzed by monitoring their competition with a fluorescent reporter ligand. We picked FP because polarization anisotropy directly gave the absolute bound fraction of the reporter ligand. We complemented it with TIRF because TIRF had phenomenal detection sensitivity, and could be used to check the FP results for consistency. We measured the binding kinetics of surface immobilized receptors instead of free receptors because less chemicals were needed, and reagents could be changed at any stage of the reaction. Our fast screening of ginsenosides made use of those attributes. Moreover, micro-array type assay could be developed from the surface reaction format.

The FP-TIRF setup and the experimental procedures were described in detail in the previous chapter. In this chapter, the results of the following experiments will be
reported and discussed, (1) characterization of the setup, (2) characterization of the tether, (3) binding kinetics of ERα with fluorescent as well as dark ligands, and (4) screening of ginsenosides for binding with ERα and PPARγ.

4.1 Characterization of the FP-TIRF setup

In this section, the results of the characterization of the apparatus will be reported under the following headings, (1) correcting for the imbalance between the // and ⊥ arms of the FP measurement, (2) monitoring the concentration of the fluorescent ligand [F], and (3) establishing the consistency between FP and TIRF results.

4.1.1 Correcting for // and ⊥ arm imbalance

Anisotropy $A$ in FP measurements is defined by Eq. (3.1) in the previous chapter. Eq. (3.1) assumes that the // and ⊥ arms of the optics are perfectly balanced, so that randomly polarized emissions from the fluorophore will give zero anisotropy value. In practice, however, the two arms are not perfectly balanced, leading to a deviated value measured. It is conventional to weigh $I_\perp$ by a correction factor, the so-called $G$-factor, to correct for the imbalance. $A$ is now given by,

$$A = \frac{I_\parallel - G \cdot I_\perp}{I_\parallel + 2 \cdot G \cdot I_\perp} \quad (4.1)$$

As explained in Chapter 3, by using a low polarization standard in the FP Standardization Kit, $G$ was found to be 0.95. The fact that $G$ was close to 1 indicated
that the two arms were almost balanced to begin with. By using a high polarization standard, we measured an $A$ of 960 mA when it was supposed to be 970 mA or higher [43]. The very small deviation lent confidence to the reliability of our FP results. Moreover, we measured the minimum and maximum $A$ of the fluorescent ligand $F$ used in binding kinetics by different means in Chapter 3. These values were all consistent, which again lent confidence to our anisotropy measurements.

4.1.2 Monitoring $[F]$

We monitored $[F]$ by measuring the bulk fluorescence. While knowing $[F]$ at any time $t$ was useful in the kinetics modeling, it was particularly useful during $F$ injections when $[F]$ varied significantly over a short time. We showed that the experimentally measured total fluorescence $I_{total}$, as given by Eq. (3.2), could be fitted to a sigmoidal function,

$$I_{total}(t) = I_{equil} \cdot \left( \frac{1 - \frac{1}{1 + \left( \frac{t}{\tau} \right)^p}}{1} \right),$$

(4.2)

where $I_{equil}$ was the equilibrium BF intensity, and $\tau$ and $P$ were fitting parameters. A typical fit is shown in Figure 4.1. In our experiments, $P$ ranged between 2 and 4, and $\tau$ ranged from a few to less than 10 s. We found that 10 s after injection, $I_{total}$ was very stable, with variation $<10\%$. This showed that the fluid inflow had stopped and the sample channel was effectively a static cell from then on.
Figure 4.1 At time $\sim 18$ s, 10 µL of 2 nM green fluorescent ligand F was flowed into the sample channel through the entrance port. The bulk fluorescence signal (open circles) was fitted to a sigmoidal function (solid curve). Inset shows schematics of the flow channel.

4.1.3 Consistency of FP and TIRF signal

We checked the consistency of FP and TIRF results by flowing F onto tethered ERα, allowed equilibration, and then flowed in excess 4OHT to compete with F. All the while, FP and TIRF signal were recorded as functions of time. The results are shown in Figure 4.2. The agreement of the two signals speaks for their reliability.
Figure 4.2  Consistency of fluorescence polarization anisotropy (FA) and TIRF signal. 2 nM F was injected at $t = 0$ and 20 μM 4OHT was injected at $t = 900$ s while FA signal (open circles) and TIRF signal (open triangles) were captured simultaneously.

As can be seen from Figure 4.2, the TIRF trace had slightly higher S/N. Nonetheless, we measured binding kinetics primarily by FP because it gave the absolute bound fraction $f$ of F once anisotropy $A$ was measured, by means of Eq. (3.3). Together with $[F]$ deduced from $I_{total}$, we could determine the on and off –rates of ERα-F interactions as well as the ERα coverage. This will be illustrated in a later section. In contrast, the TIRF signal sat on a D.C. offset attributed to the fluorescence background of free F in solution. This background varied from experiment to experiment and could not be easily determined. Even with this ill-defined background subtracted, the net TIRF signal would only give the relative population of surface-bound F. For these reasons,
we used the TIRF signal more for consistency checks than for determining kinetics rates.

4.2 Characterization of each tether link

The immobilization of functional ERα on a quartz surface was not straightforward. Standard protocols did not exist. We experimented with many approaches, from simple physi-sorption to covalently bonding the amine group of the protein receptor to silica (quartz) via 3-glycidoxy-propyl-tri-methoxy-silane. The tether was either too weak, or too irreproducible, or engendered too much non-specific binding, or the tethered receptor was not functional.

We finally arrived at a coating protocol that was relatively simple, and could immobilize receptors firmly and reproducibly without affecting their function, and yet the entire coating could be easily removed for slide recycling. Moreover, the coated surface had minimal non-specific binding for ERα or ligands.

We first coated negatively charged quartz surface (at pH 7.5) with positively charged poly-L-lysine (PLL, pI = 9.2). The electrostatic bond resulted in a firmly adsorbed monolayer of PLL. If desired, e.g., for slide recycling, this layer could be readily removed by sonication in KOH. This hydrophilic PLL layer provided a functional amine group (NH₃) for permanent crosslink to a primary antibody.

The conventional cross-linking of carboxyl groups and amine groups was initiated by the activation of the carboxyl groups with EDC/NHS, followed by cross-linking with amine groups [54]. Therefore, we premixed antibody with EDC/NHS, and allowed the carboxyl groups of the antibody to be activated by EDC/NHS in solution. The activated
and negatively-charged antibody was then flowed into the sample channel to adsorb onto positively-charged amine groups of PLL on the surface. The permanent chemical crosslink was formed gradually on surface. Crosslinking between negatively charged antibodies was less favored.

After the primary antibody was tethered, the remaining activated functional groups on the surface were passivated by excess Tris-buffered saline (TBS). A secondary antibody was then incubated, to be captured by the primary antibody. The secondary antibody was chosen to bind the N-terminus (AF1) of ERα. AF1 was furthest from the LBD. We believe the inserted layer of the secondary antibody served many useful purposes. First, it kept the tethered ERα further away from the substrate surface (about 10 nm) to minimize steric constraints. Second, the ERα epitope was well separated from its critical domains of ligand and co-regulator recruitment to guarantee functionality. Third, the extra link in the tether chain would add randomness to the dipole orientation of the bound fluorophores.

In the following sections, we will show (1) that the entire tether chain was firm and specific, (2) that the ERα functionality was preserved; and (3) that of bound F was sufficiently random.

4.2.1 Strength and specificity of tether

We first tested the adhesion of the PLL layer. We flowed Cy3B-labeled poly-L-lysine (PLL, 150 – 300 kD) at 0.1 mg/mL in PBS (pH 7.4) onto the bare quartz surface of the sample channel. The TIRF signal increased sharply and then leveled in
one min, as shown in Figure 4.3. At $t = 320$ s, we flowed in Cy3B-labeled PLL a second time. The TIRF signal increased only slightly, as can be seen from Figure 4.3. This saturation indicated that positively charged PLL adsorbed onto negatively charged quartz as a monolayer. After $t = 600$ s, the sample channel was flushed repeatedly with blank buffer. The TIRF signal remained constant, illustrating the firmness of the PLL coating. We did a control using free Cy3B alone. The TIRF signal was practically zero after a single wash (data not shown).

**Figure 4.3** Firmness of PLL coating. Cy3B-labeled poly-lysine (150 – 300 kD) at pH 7.4 was flowed onto bare quartz at $t = 0$ and 380 s. Multiple blank washes after 600 s.
Next, we tested the strength and specificity of the tether chain up to the secondary antibody Abα (see Figure 3.3). We flowed Cy3B labeled AbR (Cy3B-AbR) at 0.02 mg/mL in PBS (pH 7.4) into a sample channel coated with PLL-AbR-Abα. The TIRF signal increased sharply (due to rise in solution background fluorescence) and then more gradually (due to specific binding of Cy3B-AbR to the tethered Abα), as shown in Figure 4.4, open circles. Signal almost leveled after 10 min. The channel was then washed repeatedly with blank buffer. The TIRF signal firstly dropped abruptly as the free fluorophore in solution was flushed out, and then remained constant. This showed the firmness of tether chain. The experiment was repeated using another channel whose surface was coated with PLL-AbR without Abα. The TIRF was due only to solution background fluorescence which was removed instantly by a single wash, as shown in the same figure, ×. This demonstrated the specificity of the AbR-Abα binding.
Figure 4.4  Specificity and strength of Abα tethering. Cy3B-labeled anti-rabbit IgG (Cy3B-AbR) was flowed onto tethered Abα at $t = 0$ and washed with blank buffer at $t = 600$ s (open circles). Cy3B-AbR was flowed onto a similar surface but without Abα at $t = 0$ and washed with blank buffer at $t = 320$ s ($\times$).

We then examined the specific capture of ERα by tethered Abα. A mixture of ERα and F was flowed into two sample channels, one coated with PLL-AbR-Abα and the other coated with PLL-AbR but without Abα. The TIRF signal of F was monitored. It was found that the signal of the first channel was more than 10 $\times$ that of the second, as shown in Figure 4.5. This implied that over 90% of the ERα on the surface was specifically captured by Abα. We also flushed the first channel repeatedly with blank
binding buffer and then refilled it with solutions of F. From the FP signal, we deduced that the ERα coverage was unchanged (see a later section). This showed that the ERα was firmly tethered.

![Graph showing TIRF of green fluorescent ligand F for two tethering cases: when Abα was tethered (open circles), and when it was not (open triangles). In both cases, the same mixture of ERα and F was flowed in at t = 0. About half of the open triangle signal was due to free F in solution.](image)

**Figure 4.5** ERα tethering specificity. TIRF of green fluorescent ligand F for two tethering cases: when Abα was tethered (open circles), and when it was not (open triangles). In both cases, the same mixture of ERα and F was flowed in at $t = 0$. About half of the open triangle signal was due to free F in solution.
4.2.2 Tethering preserved receptor function

To demonstrate that our tethering scheme preserved the ligand binding function of the protein receptor, we did two tests. The first was to check if the tethered ERα would still properly recruit or block co-activators after ligand binding. The second was to compare the ERα-F binding kinetics of tethered ERα against that of free ERα in solution. Results of the first test will be reported in this section. Results of the second test will be reported in a later section.

It is known that upon binding with an agonist ligand, helix-12 (H12) of the LBD of ERα will fold in such a way to recruit the co-activator (CoA) (see Figure 2.4). In contrast, upon antagonist binding, the H12 flips outward to a position that blocks the binding of the CoA (see Figure 2.4). We ascertained the proper recruitment of CoA by measuring the TIRF signal of a fluorescent CoA (Panvera P2993) upon agonist E2 binding, versus unliganded (apo) or antagonist 4OHT binding. The result is shown in Figure 4.6. As can be seen, for apo and 4OHT-bound ERα, the signal due to recruited CoA (TIRF signal – 300 counts due to CoA in binding buffer) was no more than a hundred counts. For the case when a mixture of E2 and CoA was flowed onto tethered ERα-4OHT complex, TIRF signal due to recruited CoA gradually increased to more than four hundred counts above the background, presumably because E2 gradually displaced 4OHT. This showed that the tethered ERα was functional as far as the proper recruitment of CoA was concerned.
Figure 4.6  Proper recruitment of fluorescent co-activator CoA.  Three different cases are shown: CoA flowed onto tethered apo ERα (open triangles), mixture of CoA and antagonist 4OHT flowed onto tethered ERα (open squares), and mixture of CoA and agonist E2 flowed onto tethered ERα pre-complexed with 4OHT (open circles). Reagents were injected at $t = 0$.  At equilibrium, free CoA in solution accounted for about 300 counts of the signal.
4.2.3 Tethering preserved the random orientation of $\mu$

As mentioned in Section 3.8, for ER$\alpha$-F binding in bulk solution, the dipole $\mu$ of the fluorescent ligand F was pointing randomly for both free and complexed F. Accordingly, the total fluorescence intensity can be shown to be given by $I_{\text{total}} = I_{\parallel} + 2I_{\perp}$, Eq. (3.2); and the polarization anisotropy $A$ can be converted to the absolute bound fraction $f$ of the fluorescent ligand using Eq. (3.3). However, if ER$\alpha$ is tethered, $\mu$ of complexed F may not point randomly and Eq. (3.2) and (3.3) may not be valid.

We therefore wanted to establish the random orientation of $\mu$ for tethered ER$\alpha$-F in order to justify our use of Eq. (3.2) and (3.3) in all of our data analysis and modeling. The experiment was done as follows. 1 nM ER$\alpha$ and 1.2 nM F in binding buffer was premixed and incubated in Eppendorf tube at RT for 15 minutes in order to reach steady-state. 15 $\mu$L of the equilibrated complex was flowed into an Ab$\alpha$ coated sample channel. The polarization anisotropy $A$ (open circles) and the TIRF signal (+) were measured as functions of time, as shown in the left panel of Figure 4.7. The TIRF signal increased gradually to reach steady-state, indicating the gradual capture of ER$\alpha$-F by Ab$\alpha$. Initially, ER$\alpha$-F was freely moving in solution. Gradually, the complex was surface immobilized. Correspondingly, the dipole $\mu$ of ER$\alpha$-F was freely and randomly oriented initially, and soon was locked in place. Interestingly, $A$ (open circles) remained constant throughout the event. That implies if $\mu$ was pointing randomly at first, it must remain randomly pointing even when immobilized.

We did a follow up experiment to double confirm our conclusion. After the ER$\alpha$-F complex was immobilized on the surface, 10 $\mu$M genistein in binding buffer was
injected into the sample channel to displace the bound F from the tethered ERα. The total fluorescence intensity $I_{total}$ [Eq. (3.2) assumed] and anisotropy $A$ were measured as functions of time, as shown in the right panel of Figure 4.7. As can be seen, $A$ (open circles) decreased gradually and leveled, indicating the displacement of F by genistein. But $I_{total}$ (open triangles) remained unchanged. This is to be expected if Eq. (3.2) ($I_{total} = I_{//} + 2I_{\perp}$) described the total fluorescence intensity of the initial immobilized F as well as the final freed F, and that $I_{total}$ was proportional to the total F population in the detection volume. This again is consistent with our earlier conclusion that $\mu$ of F was randomly oriented even when immobilized on the surface.
**Figure 4.7** Effect of tethering on fluorescence anisotropy. Left panel: 15 μL of equilibrated mixture of 1.2 nM F and 1 nM ERα was flowed onto tethered Abα at $t = 0$. TIRF (+) and fluorescence anisotropy $A$ (open circles) were plotted. Right panel: When the immobilization of ERα-F reached steady state, 10 μM genistein was injected into the cell. Time was reset to a new zero then. $I_{total}$ (open triangles) and $A$ (open circles) were plotted.
4.3 Binding kinetics of the fluorescent ligand F with ERα

We studied the binding kinetics of ERα and the green fluorescent ligand F. We did the experiment as described in section 3.7.1. Based on the measured anisotropy \( A(t) \) at various time \( t \), we computed the bound fraction \( f(t) \) of F using Eq. (3.3). The bound fraction \( f(t) \) is plotted in Figure 4.8. As mentioned in previous sections, the extent of photobleaching was measured. The bleaching time constants for the association and dissociation phase were found to be 21 and 4.2 minutes respectively. This was with continuous laser irradiation. Although the photobleaching effect was small (< 10%), we still accounted for it by suitably scaling the data points.

We then fit the data using the kinetics model described in section 3.9 to determine the on and off–rates of ERα-F. The off-rate \( k_{off} \) was determined as follows. At \( t = 1,200 \) s, 20 µM E2 was injected. This huge excess of E2 prevented F from binding with apo ERα, so the decay in bound fraction \( f \) was due entirely to the one-way dissociation of F from ERα. Using Eq. (3.5), the single unknown \( k_{off} \) could be determined by best-fitting the empirical \( f(t) \).

Next, we wanted to determine the following two unknowns, the receptor coverage \( \sigma \) and the dissociation constant \( K_D (= k_{off} / k_{on}) \) in Eq. (3.5). This was done by adjusting the values of the two parameters in the kinetics model to best fit the four steady-state \( f \)'s at \( t = 200, 400, 800, \) and 900 s. This is equivalent to finding two unknowns but given four equations. The over-determination allowed us to best fit the parameters.

Finally, we plugged the values of \( \sigma, k_{off} \) and \( k_{on} \) in the kinetics model to generate the association curve, without any adjustable parameter now. The simulated curve was
compared against the empirical $f(t)$ for $t < 30$ s. A good fit lent extra confidence to the kinetics modeling. We should also point out that the sigmoidal fit of the F influx described in Section 4.1.2 (see Figure 4.1) was adopted when modeling the association phase.

To illustrate the sensitivity of the fit, values of $k_{off}$ and $k_{on}$ were set above or below the best-fit values to test their effects on the fitting. This is also shown in Figure 4.8. A 50% higher $k_{off}$ under-estimated the equilibrated bound fractions (curves labeled d) while a 50% lower $k_{off}$ over-estimated them (curves labeled c). If both $k_{off}$ and $k_{on}$ were 50% higher, F signal would increase too fast (curve a) and decay too soon (curve f). The exact opposite would occur if both rate constants were 50% lower than the best-fit values (curves b and e).
Figure 4.8  ERα-F binding kinetics. The bound fraction of F as deduced from fluorescence anisotropy was plotted against time as different reagents were injected at various time points. At time $t = 0$, 10 µL of 3 nM F was injected into the sample to replace the initial blank buffer and the bound fraction was plotted (open circles). At $t = 350$ s, 15 µL of blank buffer was injected and the equilibrated bound fraction was shown (open triangles). The last step was repeated at $t = 620$ s, and the equilibrated bound fraction was plotted (+). At $t = 900$ s, 10 µL of 3 nM F was injected and the equilibrated bound fraction was plotted (open squares). At $t = 1,180$ s, 15 µL of 20 µM E2 was injected and the subsequent dissociation of F from ERα was shown ($\times$). The steady-state signal shown was the average over ten or more neighboring spots on the sample slide. The standard deviation was about the size of the data symbols. The kinetic rate constants could be determined by best-fitting the experimental data points (bold curves). A 50% higher $k_{off}$ under-estimated the equilibrated bound fractions (curves labeled d) while a 50% lower $k_{off}$ over-estimated them (curves labeled c). If
both $k_{\text{off}}$ and $k_{\text{on}}$ were 50% higher, F signal would increase too fast (curve a) and decay too soon (curve f). The exact opposite would occur if both rate constants were 50% lower than the best-fit values (curves b and e).

Besides the very satisfactory fitting as shown in Figure 4.8, two further observations should be mentioned. First, we assumed time-independent receptor coverage $\sigma$ in the kinetics model. The good fit showed that this is a valid assumption. This implied that the receptor coverage remained the same despite the many cycles of reagent injections, which speaks once again for the strength of the tether. Second, we assumed rapid mixing in the kinetics model. The good fit lent support. The coverage $\sigma$ was one of the unknowns whose value was determined in the modeling. It was found to be about 30 molecules $\mu$m$^{-2}$. According to Eq. (3.8), the concentration of released F, relative to the background [F], was only about 2%. The low coverage therefore justified the rapid mixing assumption. In other words, the kinetics model was self-consistent. A more rigorous discussion of the rapid mixing assumption is given in the next section.

We investigated the reproducibility of the kinetics data. Given the fragility of proteins and the complexity of functional coating, we found that experiments done during the same session using the same channel or at least using the same slide would guarantee better reproducibility. However, to demonstrate the robustness of our method, we intentionally did more than 3 sets of the ER$\alpha$-F binding experiment on different slides in different sessions and mostly on different days. The average association rate $k_{\text{on}}$ and dissociation rate $k_{\text{off}}$, and their standard derivations, were listed in Table 4.1. The uncertainties were within 20%.
We also ascertained that tethering did not affect receptor-ligand binding kinetics. Two experiments were designed to show that both the dissociation constant $K_D$ and off-rate $k_{off}$ of ER$\alpha$-F binding were preserved. The results were shown in Figure 4.9. The first experiment was done by rapidly injecting a mixture of ER$\alpha$-F complex and excess E2 into a PEG-coated channel. By modeling the dissociation kinetics, the decaying trend of the bound fraction in bulk reaction could be best-fitted to determine the off-rate $k_{off}$. In another experiment, ER$\alpha$-F equilibrium complexes were injected into Ab$\alpha$-coated channel, TIRF signal increased as the ER$\alpha$-F complex gradually tethered on the surface. However, the bound fraction was found to remain unchanged. This implied that the dissociation constant $K_D$ was preserved upon ER$\alpha$ tethering. Three steady-state bound fractions between injections were measured. By modeling the binding kinetics, these steady-state bound fractions could be best-fitted to give $K_D$. The rate constants $k_{on}$ and $k_{off}$ were then deduced to be $6.8 \times 10^7$ M$^{-1}$ s$^{-1}$ and $2.5 \times 10^{-2}$ s$^{-1}$ respectively. The relative uncertainties were within 20 %. These values were consistent with those determined from tethered receptor experiments.
Figure 4.9  To illustrate that tethering did not affect receptor-ligand binding kinetics. Left panel: 15 µL of equilibrated mixture of 1 nM F and 1 nM ERα was flowed onto tethered Abα at t= 0. TIRF signal (+) and bound fraction (open circles) deduced from A were plotted. Cell was washed with 15 µL of blank buffer at t = 400 s. Subsequent steady-state bound fraction (open triangles) was deduced from A. 10 µL of 1 nM F was injected at t = 600 s and subsequent steady-state bound fraction (open squares) was again deduced from A. By modeling the ERα-F binding kinetics, the three steady-state bound fractions could be best-fitted (solid curves) to determine $K_D$. Right panel: Mixture of 1 nM F and 1 nM ERα was flowed onto PEG coated slide at t= 0. TIRF signal (open triangles) and bound fraction (open circles) deduced from A were plotted. Solution was rapidly mixed with 20 µM E2 at t= 320 s. TIRF signal (open squares) and bound
fraction (×) deduced from A were again plotted. By modeling the ERα-F dissociation kinetics, the decaying trend of the bound fraction could be best-fitted (solid curve) to determine $k_{off}$. Both panels share the same y-axis.

4.4 Justify rapid mixing assumption

In the previous section, the typical receptor coverage $\sigma$ was found to be about 30 molecules $\mu$m$^{-2}$. According to Eq. (3.8), the concentration of released F, relative to the background [F], was only about 2%. The low coverage justified the rapid mixing assumption. Moreover, the satisfactory modeling of the empirical data and the agreement of kinetics rates between tethered and free receptors lent further confidence. Nonetheless, we will demonstrate a more rigorous justification of the rapid mixing assumption in this section.

In our case of a static cell, mass transport is due solely to diffusion, and the continuity equation becomes [32],

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial z^2},$$

(4.3)

where $C$ is the free ligand concentration, $t$ is the time, $D$ is the diffusion coefficient of the ligand and $z$ is the vertical distance from the receptor tethered surface.

We will consider a simplified dissociation experiment when blank buffer is injected over tethered ERα-F and the flow soon ceased. Right after the blank flush, there is zero free F in solution. Gradually, bound F’s start to dissociate from the tethered receptors and diffuse into the solution, thus enriching the layer near the surface. In other words, $C$ now depends on $z$. Eventually, the system will reach equilibrium and $C$
becomes a constant independent of \( t \) and \( z \). We will want to compute \( C(t, z) \) to see if mixing is rapid enough.

In order to compute \( C(t, z) \), we will solve Eq. (4.3) subject to two boundary conditions (B.C.). The first B.C. is the surface flux which is the binding flux minus the dissociation flux,

\[
\text{surface flux} = D \frac{\partial C}{\partial z} \bigg|_{z=0} = k_{\text{on}}CR - k_{\text{off}}B. \tag{4.4}
\]

In Eq. (4.4), \( R \) is the area density of apo receptors, and \( B \) is the area density of bound ligand F. The second B.C. is that there should be zero concentration gradient at the middle \( (z = h/2) \) of the cell because identical binding events occur at the coverslip and the quartz slide surfaces.

\[
\frac{\partial C}{\partial z} \bigg|_{z=h/2} = 0. \tag{4.5}
\]

Eqs. (4.3) through (4.5) were solved numerically using MathCAD. Actual experimental parameters were used. They included the receptor coverage \( \sigma \) of ER\( \alpha \) (27 molecules \( \mu m^{-2} \)), rate constants and diffusion coefficient \( (D = 1.97 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}) \) of F, and height of the sample cell (~30 \( \mu m \)). The computed \( C(t, z) \) was shown in Figure 4.10. As can be seen, the maximum concentration difference between \( z = 0 \) and \( z = h/2 \) was reached after about 1 s. It was less than 2\%, in agreement with our earlier estimate based on Eq. (3.8). Afterwards, the concentration difference diminished.

When the receptor coverage was increased to the typical SPR value of about 7,000 molecules \( \mu m^{-2} \), the computed \( C(t, z) \) would become that shown in Figure 4.11. As can be seen, the concentration difference could reach 50\%, and the rapid mixing assumption would not be valid anymore.
Figure 4.10  Simulated concentration difference of ligand relative to that at half height, plotted against vertical distance from tethered surface; for different time points. Receptor coverage was 27 $\mu$m$^{-2}$. Height of sample cell was 30 $\mu$m.
Figure 4.11  Simulated concentration difference of ligand relative to that at half height, plotted against vertical distance from tethered surface; for different time points. Receptor coverage was 7,000 μm². Height of sample cell was 30 μm.
4.5 Binding kinetics of nonfluorescent ligands with ERα

We could determine the binding kinetics of a nonfluorescent ligand L by monitoring its competition with the fluorescent ligand F. Both ligands competed for the same ERα LBD. The result is shown in Figure 4.12, when the competition of the dark ligand genistein with F for ERα was used as an example. The time course could be divided into two phases, the ERα-F association phase for \( t < 620 \) s; and the L-F competition phase for \( t > 620 \) s.

During the first phase, F was flowed onto tethered ERα. ERα-F associated and reached steady-state in about 300 s. At \( t = 340 \) s, a solution of F was flowed in again and the system was allowed to equilibrate a second time. By adjusting the values of \( k_{on} \) and \( k_{off} \) for the ERα-F binding, as well as the ERα coverage \( \sigma \), we best-fitted (1) the shape of the association curve, and (2) the two steady-state bound fractions. The rate constants were found to be consistent with the values listed in Table 4.1.

During the second phase, the genistein ligand L was flowed into the channel to compete with dissociated F. This caused the bound fraction \( f \) of F to decay to a new equilibrium value for \( t > 1,000 \) s. Since the rate constants of ERα-F in Eq. (3.6) were already known from the first phase, by fitting the equilibrium \( f \) at \( t > 1,000 \) s, we could determine the dissociation constant \( K_L \) (\( = \frac{\tilde{k}_{off}}{\tilde{k}_{on}} \)) of the ERα-L binding in Eq. (3.7). We could also determine \( \tilde{k}_{off} \) of ERα-L by best-fitting the shape of the \( f \) decay in Eq. (3.7). As a result, both \( \tilde{k}_{on} \) and \( \tilde{k}_{off} \) of ERα-L could be determined.

For the second phase, the sensitivity of the fitting depends on \([L]\). If \([L]\) was too
high, the final $f$ became low and would change little with $\tilde{k}_{on}$ and $\tilde{k}_{off}$. On the contrary, if $[L]$ was too low, $f$ stayed high and the shape of the $f$ decay curve was not well defined. The optimal $[L]$ was when $f$ dropped to about 0.2 – 0.3 in the end. It could be easily identified by injecting solutions of low $[L]$ to start with. Based on that result, $[L]$ could be adjusted up. We found from experience that this trial-and-error approach was practical because all the standard dark ligands we studied have dissociation rates lower than that of F. Their displacement of F was on the minute scale. We could thus afford to skip taking kinetics data for the first ten seconds or so.

Once the optimal $[L]$ was identified, the fitting of the data in the second phase could be quite sensitive. This was illustrated in Fig. 4.12. A 50% higher $\tilde{k}_{off}$ visibly under-estimated the equilibrated bound fraction (curve labeled d) while a 50% lower $\tilde{k}_{off}$ over-estimated it (curve c). If both $\tilde{k}_{off}$ and $\tilde{k}_{on}$ were 50% higher, F signal would decay too fast (curve b). The exact opposite would occur if both rate constants were 50% lower than the best-fit values (curves a).
Figure 4.12  ERα-genistein binding kinetics. The bound fraction of F as deduced from A was plotted against time while different reagents were injected at various time points. At $t = 0$, 10 μL of 1 nM F was flowed into the sample cell (open circles). At $t = 340$ s, another 10 μL of 1 nM F was flowed in and the equilibrated bound fraction was shown (open triangles). At $t = 620$ s, 15 μL of 1 μM genistein was flowed in and the subsequent dissociation of F from ERα was shown (×). At $t = 1,250$ s, 15 μL of 10 μM E2 was flowed in to generate the baseline. The error bar of the steady-state signal was about the size of the data symbols. For the data shown, photo-bleaching was found to be negligible. The rate constants of the ERα-F binding kinetics could be determined by best-fitting the experimental data points for $t < 620$ s (bold curves). For the ERα-genistein complex, the rate constants could be determined by best-fitting the remaining experimental data points (bold decay curve). A 50% higher $k_{off}$ visibly
under-estimated the equilibrated bound fraction (curve labeled d) while a 50% lower $k_{off}$ over-estimated it (curve c). If both $k_{off}$ and $k_{on}$ were 50% higher, F signal would decay too fast (curve b). The exact opposite would occur if both rate constants were 50% lower than the best-fit values (curves a).

Besides genistein, five other standard ligands of ERα were studied. They were E2, DES, TAM, RAL and 4OHT. Their kinetics rate constants in ERα binding were listed in Table 4.1. The reproducibility and robustness of our measurements were again demonstrated by having three or more sets of results of experiments performed in different sessions using different slides. The average $\tilde{k}_{on}$ and $\tilde{k}_{off}$, together with the standard derivation, were listed in the Table 4.1. Most uncertainties were within 30%.

As far as we know, the values of the kinetics rate constants listed in Table 4.1 were known only for the first time. To date, none were reported in the literature. The nearest that we could find were rate constants measured by Rich et al (2002) [33] but they used the ligand binding domain of ERα while we used the full length. Their results were also given in Table 4.1 for easy comparison. As can be seen, except for E2, the rate constants differed very significantly.

Later on, we will comment on the discrepancy between our results and those of Rich 2002. At this time, it is useful to point out that equilibrium binding affinity data (instead of kinetics rate constants) were much more available in the literature. They were usually reported in terms of Relative Binding Affinity (RBA) for comparison of results across laboratories [2]. RBA is the binding affinity of ERα-L relative to ERα-E2. It could be computed once the equilibrium dissociation constant $K_L$, or half maximal
inhibitory concentration IC₅₀ is known, by using the following equations,

\[ K_L = \frac{IC_{50 \ for \ L}}{1 + \frac{[F]_{\text{total}}}{K_D}}, \quad (4.6) \]

and,

\[ RBA = \frac{IC_{50 \ for \ estradiol}}{IC_{50 \ for \ L}} \times 100, \quad (4.7) \]

where \([F]_{\text{total}}\) is the total (free + bound) concentration of the fluorescent ligand F used in the competition, and \(K_D (= k_{\text{off}}/k_{\text{on}})\) is the equilibrium dissociation constant of ERα-F [2].

In our case, \(K_L = \frac{\tilde{k}_{\text{off}}}{\tilde{k}_{\text{on}}}\) (listed in Table 4.1) and IC₅₀ for L could be computed using Eq. (4.6). Accordingly, RBA of L could be computed using Eq. (4.7), and the results are listed in Table 4.1.

RBA values were reported by other groups. Among them, we selected those using full length human ERα and fluorescence polarization assays (hERα-FP) [44, 55, 56, 57, 58, 59, 60]. We averaged the reported values and computed their standard deviations. They are also tabulated in Table 4.1 for easy comparison. As can be seen, our RBA values were consistent with the reported ones.

We could also compute the RBA values based on the results of Rich 2002. These were shown in Table 4.1. They were very different from the other reported values. Rich 2002 used SPR to assay the binding of ERα(LBD only) with its ligands, while all the others used FP to study full length ERα; that may explain the difference.
<table>
<thead>
<tr>
<th>Ligand</th>
<th>$k_{on}$ (10^5 M⁻¹ s⁻¹)</th>
<th>$k_{off}$ (10⁻³ s⁻¹)</th>
<th>$K_D$ (nM)</th>
<th>RBA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>This work</td>
<td>Rich 2002</td>
<td>This work</td>
<td>Rich 2002</td>
</tr>
<tr>
<td><strong>Agonist</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>green F</td>
<td>570 (100)</td>
<td>---</td>
<td>30 (5)</td>
<td>---</td>
</tr>
<tr>
<td>E2</td>
<td>11 (3)</td>
<td>13 (6)</td>
<td>1.5 (4)</td>
<td>1.2 (2)</td>
</tr>
<tr>
<td>DES</td>
<td>10 (1)</td>
<td>60 (7)</td>
<td>1.5 (3)</td>
<td>0.05 (2)</td>
</tr>
<tr>
<td>GEN</td>
<td>0.26 (8)</td>
<td>---</td>
<td>2.3 (5)</td>
<td>---</td>
</tr>
<tr>
<td><strong>Antagonist</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAM</td>
<td>1.0 (3)</td>
<td>0.045 (1)</td>
<td>1.73 (6)</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td>4OHT</td>
<td>2.4 (9)</td>
<td>0.023 (1)</td>
<td>1.6 (4)</td>
<td>0.041 (1)</td>
</tr>
<tr>
<td>RAL</td>
<td>3.3 (5)</td>
<td>---</td>
<td>1.3 (5)</td>
<td>---</td>
</tr>
</tbody>
</table>

| **Table 4.1** Rate constants, dissociation constants and relative binding affinity (RBA) for ERα-ligand interactions. Results of this work are compared against values reported in Rich 2002 [33] and others hERα-FP [44, 55, 56, 57, 58, 59, 60]. ER ligands are estradiol (E2), diethylstilbestrol (DES), genistein (GEN), tamoxifen (TAM), 4-hydroxytamoxifen (4OHT) and raloxifene (RAL). |
| * In the binding of raloxifene with hERα, all published RBA values were measured using radio-assays [61, 62, 63]. No FP results were reported. |

Our results showed that $k_{off}$ of the various nonfluorescent ligands were similar. Their difference in binding affinity was due to different $k_{on}$. How that might be explained by their chemistry (structure and reactivity) will be explored in Section 4.9.
4.6 Positive and negative controls

In the previous section, we showed that all the known ERα ligands that we have studied (E2, DES, genistein, TAM, 4OHT and RAL) did bind to ERα and competed with F for ERα. In other words, all the positive results that we saw were true positives. We did not see any false positives.

We also checked for false negatives. We tested testosterone (TEST) and dexamethasone (DEX) for binding with ERα. It is known that these two ligands are not ligands of ERα. The test results are shown in Figures 4.13. As can be seen, no binding was detected. In other words, the negative results were true, no false negatives were seen.
Figure 4.13  Negative and positive controls of ERα ligand binding. Top: Tethered ERα was first complexed with F. As negative control, non-ERα ligand dexamethasone (DEX) at 10 μM was injected into sample cell at time $t = 0$ and fluorescence anisotropy was measured for the next 300 s. As positive control, estradiol (E2) was injected at the
end. Bottom: The experiment was repeated with another non-ERα ligand testosterone (TEST).

### 4.7 Screening ginsenosides for ERα binding

As mentioned in previous chapters, our FP-TIRF method could be used to fast-screen potential ligands of the tethered receptor. We screened a total of eleven ginsenosides for binding with tethered ERα. They were chosen based on recent reports of their steroid-like properties [64, 65]. The experimental protocol was explained in Section 3.7.3, and outlined in the caption of Figure 4.14. Briefly, each ginsenoside at high concentration was flowed onto tethered ERα-F complex. Displacement of F due to binding of ERα with the ginsenoside would show up in the decay of the bound population of F. The experiment was repeated for each of the eleven ginsenosides, and near the end, genistein was injected as positive control. Finally, excess E2 was injected to define the baseline. The entire series of experiment were repeated three times. Because the ERα coverage varied among the three runs, in order to compare data across runs, we normalized the maximum anisotropy reading of each trace to one, and defined the minimum anisotropy reading of each trace (after injection of excess E2) as zero. For each ginsenoside and control, the averaged bound population of F, so normalized, is plotted in Figure 4.14 as a function of time. The relative standard deviation was only about 3%. As can be seen, there was no measurable binding of any of the ginsenosides with ERα. The null results were double-checked using fluorescence polarization assays of ERα-F in solution [66]. The two results agreed.
Figure 4.14  Screening ginsenosides for binding with ERα. Tethered ERα was first complexed with F. 10 μM of a ginsenoside was injected into sample cell at time $t = 0$ and fluorescence anisotropy was measured for the next 300 s. The cell was refreshed by flushing with blank buffer. The injection-wash cycle was repeated with a new ginsenoside each time. As positive control, 200 nM genistein was injected in the last round. Minimum anisotropy was determined by injecting excess E2. The entire experiment was repeated three times, with the order of the ginsenosides randomized. In the third trial, the ginsenoside concentration was increased to 50 μM. The bound F population, with the maximum of each trace normalized to one, was computed. Plotted are the averages of the three data sets. The standard deviation was about twice the size of the data symbol.
4.8 Screening ginsenosides for PPARγ binding

Using the FP-TIRF system, we screened the same eleven ginsenosides for binding with surface-immobilized PPARγ (LBD). This time, we streamlined the procedure still further by simply physi-sorbing the receptor on PLL-coated channel surfaces. Binding was measured by TIRF. Results of ten ginsenosides and one positive control are shown in Figure 4.15. The other ginsenosides not shown did not exhibit detectable binding. Interestingly, two ginsenosides, Rb1 and 20(S)-Rg3, did show significant binding to PPARγ, relative to the positive control GW1929. The experiment was repeated twice and the same results were seen.

In view of the crudeness of the physi-sorption scheme, we double-checked the reliability of the screening results by repeating the experiment but tethered the PPARγ (LBD) by a surface-immobilized antibody specific for the receptor this time. The epitope was far from the ligand binding region. We measured binding using FP. Results are shown in Figure 4.16. Clearly, the same conclusion was drawn. Both the positive and negative results were double-checked using PPARγ (LBD)-F in solution [66]. The two results agreed. Downstream effects of ginsenosides on diabetic mice had since been investigated by our collaborators and very interesting results that corroborated with our in vitro binding measurements were seen [67].
Figure 4.15  Screening ginsenosides for binding with PPARγ physi-sorbed on PLL. Physi-sorbed PPARγ was first complexed with its specific F. 10 μM of a ginsenoside was injected into the sample channel at time $t = 0$ and TIRF was measured for the next 300 s. The cell was refreshed by flushing with blank buffer. The injection-wash cycle was repeated with a new ginsenoside each time. As positive control, a PPARγ standard ligand GW1929 at 10 μM was injected in the last round to generate the TIRF baseline.
Figure 4.16  Screening ginsenosides for binding with antibody-captured PPARγ. Tethered PPARγ was first complexed with a specific F. This F was different from the one used in Figure 4.15. 10 μM of a ginsenoside was injected into sample channel at time \( t = 0 \) and fluorescence anisotropy was measured for the next 300 s and converted to bound F fraction. The cell was refreshed by flushing with blank buffer. The injection-wash cycle was repeated with a new ginsenoside each time. As positive control, a PPARγ standard ligand GW9662 at 10 μM was injected in the last round to determine the minimum anisotropy.
4.9 Binding affinity and chemical structure

We attempted to identify the correlations between binding affinity and the chemical structure of the ligands. For the case of ERα, the structures of the standard ligands and the non-ligand are shown in Figure 4.17, and ranked according to their RBA. As pointed out by Pike 2006 [16], the phenolic A-ring (colored green in the figure) is common to all the ligands, but is missing in the non-ligand testosterone. It is also missing in the ginsenosides, as shown in Figure 4.18. That may explain their non-binding.

For the case of PPARγ, it is interesting to note that in our measurements; only 20(S)-Rg3 and Rb1 would bind. The other ginsenosides did not. 20(S)-Rg3 and 20(R)-Rg3 are isomers, differing only in the spatial arrangement of the hydroxyl group on the chiral carbon (C-20) (circled in blue in Figure 4.18). That difference apparently marks PPARγ ligands from non-ligands. Similarly, comparing 20(S)-Rg3 against 20(S)-Rh2, the only difference is one more sugar moiety (highlighted by red square in the figure) that is present in the former but not in the latter. That is a second feature that correlates with binding. The same additional sugar moiety is also found in the only other ligand Rb1. The hydroxyl group on C-20 is replaced by sugar moiety for Rb1 (highlighted by green rectangle in the figure). This indicates that the sugar moiety on C-20 may be a key feature. This will require further corroboration by screening more ginsenosides.

The structures of the standard ligands of PPARγ are illustrated in Figure 4.19. They are ranked by binding strength. Because tens of μM of 20(S)-Rg3 and Rb1 were
needed to compete with nM of F (see Figure 4.15 and 4.16), their binding strength would be similar to Pioglitazone. Yet, based on Figures 4.18 and 4.19, their common features are not apparent. It will be useful if molecular dynamics modeling can be performed to elucidate the observations mentioned above. The deeper principles that can be learned will certainly enhance our understanding of ligand-receptor interactions in general, and ginseng pharmacology as well as the bio-hazards of endocrine disruptors in particular.
Figure 4.17  Structural characterizations of ER ligands (top) and non-ligands (bottom).

Affinity among ER ligands decreases from top to bottom. Green colored phenolic
A-rings are the common characteristics among ER ligands. Antagonist side chain moieties are marked with purple spots. ER ligands are estradiol (E2), diethylstilbestrol (DES), genistein (GEN), tamoxifen (TAM), 4-hydroxytamoxifen (4OHT) and raloxifene (RAL) [16]. Non-ER ligands are testosterone (TEST) and dexamethasone (DEX).
Figure 4.18  Structural characterizations of Ginsenosides. Red squares and blue circles highlight the common structures among those ginsenosides that bind with PPARγ. Green rectangle highlights another possible binding feature.
Figure 4.19  Structural characterizations of PPARγ ligands.  Affinity decreases from top to bottom.  Red and blue boxes highlight the common structures.
CHAPTER 5  CONCLUSION

In this thesis work, we developed a novel method for measuring receptor-ligand binding kinetics based on FP-TIRF in a flow cell format. The project was very interdisciplinary, requiring expertise in physics as well as in biochemistry. The entire methodology, from instrumentation through data capture to kinetics modeling was worked out basically from scratch. Challenging aspects of the instrumentation included the design and implementation of the FP-TIRF optics that was built around a microscope, the design of a flow cell that was simple but very practical, and the working out of a receptor tethering scheme that was secure while preserving the receptor functions at the same time.

The design of the data capture protocol was no less demanding. The receptor coverage had to be kept low to guarantee rapid mixing, which implied very weak signal. The fragility of protein receptors necessitated carefully planned and executed data runs, together with positive and negative controls, and automated data capture for kinetics modeling. All the critical parameters had to be identified beforehand, such as [F] and [L], so that the feasibility of their accurate determination could be factored in.

Finally, the kinetics modeling part was another challenge. The simplifying assumptions, such as the random pointing of the dipole of the fluorophore and the rapid mixing of reagents, had to be clearly identified and justified based on empirical data. The robustness and the sensitivity of the model had to be tested. We also compared the FP data against the TIRF data for self-consistency. In cases when alternative assays were available, such as the measurement of equilibrium binding affinities, we
double-checked our results against those gotten with conventional techniques.

We first applied our method to measure the binding kinetics of ERα with its standard ligands. The system was chosen for its relevance in anti-estrogen therapy of breast cancer and in the screening of environmental pollutants for endocrine disruptors. While equilibrium binding affinities had been measured by many other groups, kinetics rate constants had never been reported for full length ERα. For the six standard ligands we studied, their rate constants were found to be similar. For example, their off-rates ranged from $1.3 \times 10^{-3}$ to $2.3 \times 10^{-3}$ s$^{-1}$ while their on-rates ranged from $0.3 \times 10^5$ to $11 \times 10^5$ M$^{-1}$ s$^{-1}$. Their equilibrium binding strengths, expressed in terms of RBAs, were shown to agree with results reported by others who also measured full length human recombinant ERα using FP. We found that the four times higher binding affinity of raloxifene relative to tamoxifen was due primarily to a three times higher on-rate. This kind of information will be useful for SERM development in breast cancer therapy.

We next applied our method to screen ginsenosides for binding with ERα. This was motivated by recent reports that some ginsenosides appeared to act like hormones in animal models. We demonstrated that the screening could be fast. A total of eleven ginsenosides could be screened in less than two hours. We showed that none of the ginsenosides bound to the tethered ERα, even at 50 μM concentration. We finally screened ginsenosides for binding with PPARγ. We found that none of them would bind except 20(S)-Rg3 and Rb1. The implication of their potential positive effect in diabetes management was being actively pursued by our collaborators.

Given this powerful and convenient FP-TIRF technique, much further work can be explored. For example, one can contrast the binding kinetics of full length versus
LBD ERα, in order to clarify the role of non-LBD regions in ligand interaction. Another interesting comparison is monomer versus dimer ERα. One will want to investigate the possible cooperativity among the multiple binding sites. Environmental effects of temperature, crowding, and chaperones can be studied too. Because our fluorescence technique can track multi-partners simultaneously, ligand-induced downstream effects such as co-regulator recruitment and ERE binding can be readily monitored. Based on our experience in ERα and PPARγ tethering, we should be able to extend to other receptor systems as well for a more comprehensive investigation of signaling and regulation by small molecules. These are all important basic science issues. For more practical applications, fast screening of potential endocrine disruptors will be a good example.
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