Molecular Imaging and Sensing Using Plasmonic Nanoparticles

by

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Julie Ostrander

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of Biomedical Engineering
in the Graduate School of Duke University

2010
ABSTRACT

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Abstract

Noble metal nanoparticles exhibit unique optical properties that are beneficial to a variety of applications, including molecular imaging. The large scattering cross sections of nanoparticles provide high contrast necessary for biomarkers. Unlike alternative contrast agents, nanoparticles provide refractive index sensitivity revealing information regarding the local cellular environment. Altering the shape and composition of the nanoparticle shifts the peak resonant wavelength of scattered light, allowing for implementation of multiple spectrally distinct tags. In this project, nanoparticles that scatter in different spectral windows are functionalized with various antibodies recognizing extra-cellular receptors integral to cancer progression. A hyperspectral imaging system is developed, allowing for visualization and spectral characterization of cells labeled with these conjugates. Various molecular imaging and microspectroscopy applications of plasmonic nanoparticles are then investigated. First, anti-EGFR gold nanospheres are shown to quantitatively measure receptor expression with similar performance to fluorescence assays. Second, anti-EGFR gold nanorods and novel anti-IGF-1R silver nanospheres are implemented to indicate local cellular refractive indices. Third, because biosensing capabilities of nanoparticle tags may be limited by plasmonic coupling, polarization mapping is investigated as a method to discern these effects. Fourth, plasmonic coupling is tested to monitor HER-2 dimerization. Experiments reveal the interparticle conformation of proximal HER-2
bound labels, required for plasmonic coupling-enhanced dielectric sensing. Fifth, all
three functionalized plasmonic tags are implemented simultaneously to indicate
clinically relevant cell immunophenotype information and changes in the cellular
dielectric environment. Finally, flow cytometry experiments are conducted utilizing the
anti-EGFR nanorod tag to demonstrate profiling of receptor expression distribution and
potential increased multiplexing capability.
Dedication

I dedicate this work to my wife, Caroline who has showed unwavering love and support over the past years, and our daughter, Annika.
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List of Abbreviations

Ab - Antibody
Ag – Silver
ATCC – American Type Culture Collection
Au - Gold
A.U. – Arbitrary Units
BCA - Bicinchoninic Acid
BSA – Bovine Serum Albumin
CCD - Charge-Coupled Device
CTAB - Cetyltrimethylammonium Bromide
CV - Coefficient of Variation
DAPI- 4’-6-Diamidino-2-Phenylindole
DDA – Discrete Dipole Approximation
DDSCAT - Discrete Dipole Approximation for Scattering and Absorption
DMEM – Dulbecco’s Modified Eagle’s Medium
DNA - Deoxyribonucleic Acid
EGFR – Epidermal Growth Factor Receptor
ELISA - Enzyme-Linked Immunosorbent Assay
EGF – Epidermal Growth Factor
FACS – Fluorescence-Activated Cell Sorting
FBS – Fetal Bovine Serum
FITC - Fluorescein Isothiocyanate
FWHM – Full Width Half Maximum
GAPDH - Glyceraldehyde 3-Phosphate Dehydrogenase
GFP – Green Fluorescent Protein
HAuCl$_4$ - Tetrachloroaurate trihydrate
He-Ne – Helium-Neon
HEPES - 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HER-2 – Human Epidermal Growth Factor Receptor 2
HRP - Horseradish Peroxidase
IGF-1R – Insulin-like Growth Factor 1 Receptor
IgG – Immunoglobulin G
IR – Infrared
IRS-1 – Insulin Receptor Substrate 1
LED – Light Emitting Diode
LSPR – Localized Surface Plasmon Resonance
MAPK - Mitogen-Activated Protein Kinase
MEM – Modified Eagle’s Medium
M-PER - Mammalian Protein Extraction Reagent
MPTES - Methacryloxy Propyltrimethoxysilane
ND – Neutral Density
NIH – National Institute of Health
NIR – Near-Infrared
NP - Nanoparticle
PB – Probability Binning
PBS – Phosphate Buffered Saline
PEG – Polyethylene Glycol
PI3K - Phosphoinositide 3-Kinase
PMT – Photomultiplier Tube
PTK – Protein Tyrosine Kinase
PVDF – Polyvinylidene Fluoride
QTH – Quartz Tungsten Halogen
RBS – Ribosome Binding Site
RI – Refractive Index
RIPA – Radio Immunoprecipitation Assay
RIU – Refractive Index Units
RPM – Rotations Per Minute
SAM – Self-Assembled Monolayer
SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM – Scanning Electron Microscopy
SEM – Standard Error of the Mean
STAR – Signal Transduction Assay Reaction
STD – Standard Deviation
SPR – Surface Plasmon Resonance
TBST – Tris-Buffered Saline Tween-20
TEM – Transmission Electron Microscopy
UV – Ultra-Violet
VIS - Visible
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1 Introduction

1.1 Motivation

Cancer is the second leading cause of death in the United States, preceded only by ischemic heart disease [1]. In 2010, there are projected to be over 1.5 million new cancer cases and nearly 570,000 cancer related deaths [2]. Nonetheless, there has been a 21.0% decrease since 1990 in the death rate associated with cancer, attributed mostly to a reduction in lung, prostate, breast, and colorectal cancer rates [2]. The decline in these rates is primarily due to advances in early detection techniques and improved therapies. Early detection allows for identification of the cancer while it still remains localized, thereby improving prognosis. Desirable improvements to current detection methods include improved biomarker technology capable of further discerning aggressive cancer types [3]. In the case of metastatic cancers that were not identified in early stages, significant work is still required in developing potential therapies.

Early stage cancer cells overexpress certain receptors that can be monitored to indicate immunophenotype and corresponding metastatic potential. Increased expression of these receptors may occur prior to more identifiable changes in internal cellular structure, size, or shape. Clinical studies have even shown that specific immunophenotypes confer resistance to some cancer treatments and therefore provide an indication of the best course of disease therapy. For these reasons, individual and
multiplex biomarkers for profiling of receptor expression can improve accuracy of early diagnosis and prognosis [4].

Upon identification of early stage cancers, drug therapies are often implemented that target only one step in a very complex signaling pathway. When posed with an interruption in one pathway, these cells often adapt to utilize alternative mechanisms to maintain tumorigenesis. For instance, the drug tamoxifen is effective in the treatment of breast cancer. However, cells can acquire resistance to the therapy through increased EGFR/HER-2 dimerization [5]. Co-targeting of multiple pathways simultaneously to increase therapy effectiveness requires tools to gain a better understanding of receptor interaction [6].

Resection of a malignant tumor also benefits from molecular imaging biomarkers. Without well-defined borders of the tumor cells invading healthy tissue, surgeons must rely on some method of differentiating the malignancy. Potential contrast agents including fluorophores and quantum dots are limited by cytotoxicity, although current advances in coating and encapsulation are reducing these effects [7, 8].

Plasmonic nanoparticles (NPs) represent an alternative class of biomarkers ideal for assessing receptor expression in such applications. These biocompatible contrast agents exhibit a surface plasmon resonance, which produces enhanced scattering at a peak wavelength upon excitation by incident light. Variations in shape, size, and composition greatly affect the peak scattering wavelength of these NPs, allowing for
greatly expanded multiplex applications in the 400 – 2200 nm spectral range. More importantly, the peak resonance of NPs is sensitive to the local refractive index (RI) environment extending over nanometer distances, detecting slight changes in ligand binding status, receptor dimerization, or internal cell structure. Furthermore, photothermal therapy of plasmonic NPs has even shown success in effective targeting of malignant tumors [9, 10].

1.2 Project overview

The purpose of this project is to investigate multiple plasmonic NP biomarkers for molecular imaging and sensing applications. First, characterization of these labels requires the development of a hyperspectral imaging system for a previously constructed darkfield epi-illumination microscope. Second, while NPs show promise over fluorescence biomarkers in sensing capacity, the ability to quantify receptor expression levels requires investigation. Third, plasmonic coupling complicates not only receptor quantification, but additionally dielectric sensing. Polarization mapping is therefore explored as a potential method to discern plasmonic coupling effects between NP pairs. Fourth, previous results present a technique for the observation of receptor dimerization using HER-2 antibody (Ab) gold nanospheres that requires investigation of proximal pair conformation. Fifth, novel dielectric sensing is further explored with anti-IGF-1R silver nanospheres and anti-EGFR gold nanorods. Sixth, all three developed tags
are implemented in multiplex immunophenotype profiling. Finally, the use of anti-EGFR nanorods as contrast agents in flow cytometry is studied, with the potential of greatly expanding current cytometry multiplexing capabilities.

### 1.3 Thesis outline

A detailed overview of the plasmonic NP concepts and modeling theories pertinent to the research areas explored are provided in Chapter 2. The development, characterization, and validation of the hyperspectral imaging system is presented in Chapter 3. Chapter 4 explores a comparison of NP and fluorescence based receptor quantification. The application of polarization mapping to discern plasmonic coupling modes of NP pairs is described in Chapter 5. A broad study of molecular imaging using plasmonic NPs is presented in Chapter 6. This chapter first addresses the individual use of silver nanospheres and gold nanorods for cellular environment dielectric sensing. It then explores plasmonic coupling effects of gold nanosphere pairs for monitoring of receptor dimerization. The chapter concludes with the multiplex application of all three NP tags towards immunophenotype profiling. Chapter 7 investigates the application of gold nanorods in flow cytometry. Finally, Chapter 8 presents a brief summary of all findings and future directions.
2 Background

2.1 Plasmonic nanoparticles

Noble metal NPs exhibit unique optical effects that are beneficial for a variety of applications. These properties are due to the interaction of incident electromagnetic fields with free conduction band electrons. In bulk metal, these conduction electrons are polarized relative to the heavier positive core ions [11]. The net charge restoring force results in a coherent oscillation of the conduction band electrons (Figure 2.1). This quantized plasma, or charge density wave is known as a plasmon. In the case of bulk metal, the oscillation represents a volume plasmon. However, upon introduction of a boundary of two different dielectric environments, such as a surface interface, the charge density wave is known as a surface plasmon. When referring to a NP, the plasmon is confined to a very small volume representing a localized surface plasmon. For most spherical NPs, a simple dipolar mode model adequately characterizes the oscillation. However, larger noble metal particles may have oscillations of higher mode orders due to inhomogeneous polarization. These higher mode orders are associated with lower energy levels, resulting in red shifted surface plasmon resonance peak wavelengths relative to smaller sized spherical NPs [12].
2.2 Benefits of plasmonic nanoparticles

Optical biomarkers for EGFR have the potential for both \textit{in vitro} and \textit{in vivo} imaging applications. \textit{In vitro}, such optical probes can be used to quantify receptor expression and provide information about receptor signaling processes. \textit{In vivo}, optical biomarkers can be used to assess modulation of receptor expression (as an assessment of tumor progression or response to therapy) as well as to demarcate tissue boundaries under certain circumstances, e.g., during surgery. One biomarker technique that has
recently gained attention is the use of plasmonic NPs. NPs composed of noble metals are ideal contrast agents because they exhibit a surface plasmon resonance, which produces enhanced scattering at a peak wavelength upon excitation by incident light. They also show promise over alternatives such as chemical fluorophores and quantum dots, which are limited by their cytotoxicity and photobleaching. Immunolabeled NPs (i.e., NPs having one or more antibodies attached to their surface for binding with specific targets) provide the advantages of not being cytotoxic and having a high degree of stability of optical signal over time [14, 15]. Furthermore, the scattering spectra of NPs are determined by their size, shape, and composition, which provide the flexibility to easily tailor NP biomarkers for various applications. Importantly, the scattering spectra of NPs are sensitive to the local RI environment extending over nanometer distances, which allows NPs to provide information regarding the cellular nanoenvironment. Thus, the spectra of light scattered by NPs can potentially indicate biological function (including cell signaling) or receptor-binding status [16]. For these reasons, immunolabeled NPs show promise as a powerful molecular imaging tool in vitro and may provide a platform for development of in vivo targeted-delivery strategies [17].
2.3 **Modeling of plasmonic nanoparticles**

2.3.1 **Mie theory**

Mie theory represents an exact solution to Maxwell’s equations, describing scattering and absorption of electromagnetic waves by spherical particles of any size or composition. The scattering, extinction, and absorption cross sections derived exactly through Mie theory are expressed as:

\[
\sigma_{sca} = \frac{W_s}{I_i} = \frac{2\pi}{k^2} = \sum_{n=1}^{\infty} (2n + 1)(|a_n|^2 + |b_n|^2) \quad [2.1]
\]

\[
\sigma_{ext} = \frac{W_{ext}}{I_i} = \frac{2\pi}{k^2} = \sum_{n=1}^{\infty} (2n + 1) \text{Re}\{a_n + b_n\} \quad [2.2]
\]

\[
\sigma_{abs} = \sigma_{ext} + \sigma_{sca} \quad [2.3]
\]

where \(a_n\) and \(b_n\) are the scattering coefficients, \(k\) is the wavenumber, \(W_s\) is the rate at which energy is scattered, \(W_{ext}\) is the rate at which energy undergoes extinction, and \(I_i\) is the irradiance of unpolarized light [18]. These equations represent a summation of all of the partial waves of different modes. For instance, the total Mie extinction can be broken up into dipolar, quadropolar, and higher order modes. Each order consists of surface plasmons comprising the electric modes, and eddy currents comprising the magnetic modes [19]. Because the theory encompasses multipole modes, there is no size limitation, allowing for evaluation of both Rayleigh and Mie scatterers. The scattering and absorbance cross sections can be used to determine the intensity loss of incident light through elastic scattering or absorbance. In both cases the intensity loss follows a Beer-Lambert decay relationship [19].
2.3.2 Quasistatic model

While Mie theory provides an exact solution to spherical scattering, it does not adequately describe the physical processes taking place. The quasi-static model provides an approximation for scattering from spheres much smaller than the wavelength of the incident light. In this regime, spatially the field is static with no retardation effects. Although it is not considered an electrodynamic process, there are still temporal oscillations according to \( e^{-i\omega t} \) [20]. If retardation of the electromagnetic wave occurs, then multipole mode resonance excitation will exist [21].

LaPlace’s equation, which describes the electrostatic state, provides a method of solving for the electromagnetic field surrounding the particle [22]. The equation with potential \( \Phi \) is expressed as:

\[
\nabla^2 \varphi = 0 \tag{2.4}
\]

After applying appropriate boundary conditions, one can solve for the field inside and surrounding the sphere. The electromagnetic field relates to the potential according to:

\[
E = -\nabla \varphi \tag{2.5}
\]

The scattering field outside of the sphere, as determined by this field-potential relationship, matches the dipole moment and provides the polarizability of the sphere, also known as the Clausius-Mossotti relation:

\[
\alpha = 4\pi r^3 \frac{\varepsilon_1 - \varepsilon_2}{\varepsilon_1 + \varepsilon_2} \tag{2.6}
\]
where \( r \) is the sphere radius, and \( \varepsilon_1 \) and \( \varepsilon_2 \) are the dielectric constants of the sphere and immersion media, respectively. The polarizability leads to determination of the Rayleigh scattering and absorbance, represented by the scattering and absorbance cross-sections

\[
\sigma_{sca} = \frac{k^4}{6\pi} |\alpha|^2
\]  
\[\text{[2.7]}\]

\[
\sigma_{abs} = k\text{Im}(\alpha)
\]  
\[\text{[2.8]}\]

where \( k \) is the wavenumber.

### 2.3.3 Discrete dipole approximation

The discrete dipole approximation (DDA) represents an alternative method for solving for the absorption and scattering efficiencies of a target that is not necessarily limited to spheres [23, 24]. While Mie theory and the quasistatic approach are only applicable for spherical shaped targets, the DDA allows for targets of varying geometry [25]. The Claussius-Mossotti equation, which relates the polarizability of individual atoms to the bulk dielectric properties, is used in the DDA, where the target is approximated by a polarizable point array, each representing a sub-volume of the overall target. These points acquire dipole moments from the incident electric field as well as contributions from each of the other n-1 dipoles. This approach produces an exact solution of the absorption and scattering efficiencies for the finite array, although it represents an approximation for the continuum target it is meant to represent.
Certain conditions need to be met for the approximation to be considered valid [23]. First, the lattice spacing must be small compared to the wavelength of incident light according to the following relation:

\[ |m|kd \leq 1 \]  

[2.9]

where \( m \) is the complex RI of the target material, \( k \) is the wavenumber, and \( d \) is the interdipole separation. Second, the number of dipoles must be large enough to adequately represent the target.

\[ N > \left( \frac{4\pi}{3} \right)^3 |m|^3 (ka_{eff})^3 \]  

[2.10]

where the effective radius of the target volume is represented by:

\[ a_{eff} = \left( \frac{3V}{4\pi} \right)^{\frac{1}{3}} \]  

[2.11]

and \( V \) is the target volume.

### 2.3.4 Nanorod modeling

Two methods have been used to theoretically investigate the influence of nanorod aspect ratio and the corresponding immersion medium RI on the localized surface plasmon resonance peak. The first theoretically determined relationship is based on Gans theory of elongated ellipsoids, an extension of Mie theory. Link et al. determined the relationship to be [26]:

\[ \lambda_{max} = (53.71 \cdot R - 42.29) \cdot \epsilon_m + 495.14 \]  

[2.12]
where $\epsilon_m$ represents the medium dielectric constant, which experiments indicate is also dependent on the nanorod aspect ratio.

Alternatively, Brioude et al. used discrete dipole approximations of the elongated ellipsoids to derive a similar relationship. In the calculation, 40,000 dipoles were implemented. The observed relationship was as follows [27]:

$$\lambda_{max} = (52.95 \cdot R - 41.68) \cdot \epsilon_0 + 466.38$$

[2.13]

where $\epsilon_0$ represents the medium dielectric constant.

### 2.4 Application of Plasmonic Nanoparticles

Plasmonic NPs benefit several applications, including molecular imaging, environmental sensing, and photothermal therapy. These applications take advantage of the large optical contrast associated with the increased scattering and absorption cross sections. Over the last decade, these applications have been investigated using NPs of various size, shape, and composition, each with advantages for different applications. In addition, these NPs have been conjugated to various antibodies, aptamers, and peptides [28].
2.4.1 Molecular imaging

One of the first molecular imaging studies exploring the use of plasmonic NPs evaluated the use of anti-EGFR conjugated 12 nm gold nanospheres for in vivo confocal reflectance molecular imaging of epithelium [29]. In addition, the study showed for the first time the ability to quantify the number of gold conjugates (alternative term for immunolabeled NPs) bound per cell. The protocol developed in this study has been adapted and implemented in numerous other studies. For instance, it was later implemented with anti-EGFR conjugated 35 nm gold nanospheres for diagnosis of oral cancer by El-Sayed et al. The study successfully discriminated between malignant (EGFR-expressing) and non-malignant (EGFR-non-expressing) cells [14]. This same group also developed a protocol for nanorod fabrication and conjugation that has been widely implemented. In this study, anti-EGFR conjugated nanorods were applied to molecular imaging and photothermal therapy of cancer cells [30].

These studies primarily targeted EGFR, but other works have targeted HER-2. HER-2 Ab has been conjugated to gold nanoshells for darkfield molecular imaging and photothermal therapy [31], as well as gold nanorods for photoacoustic imaging applications [32]. Literature searches show that the conjugation of HER-2 Ab to gold nanospheres has not yet been investigated. HER-2 Ab has also been conjugated to silver nanospheres for plasmon-resonant enhanced third-harmonic generation for molecular
imaging of cancer cells [33]. Literature searches show no studies in which silver nanospheres have been conjugated to any other antibodies.

Finally, anti-IGF-1R has also been conjugated to NPs, but only carbon nanotubes, not plasmonic NPs [6]. In one such study, IGF-1R and HER-2 targeted carbon nanotubes were used to kill breast cancer cells through photothermal therapy. Literature searches show that the conjugation of anti-IGF-1R to gold or silver plasmonic NPs has not been investigated previously.

2.4.2 Multiplex optical sensing

There are obvious advantages to multiplex optical sensing, including high throughput assays for the screening of multiple markers associated with different diseases. [34] Multiplexing of plasmonic NPs has already been demonstrated in optical sensing applications. One biosensor consisted of a “lab in a tube” setup that successfully observed the presence of three different species of IgG1 antigen in suspension. Upon antigen binding to three different sized nanorods of increasing aspect ratio, three peak shifts were observed indicating presence of all three species [35]. A similar setup was alternatively targeted to pathogens including E. coli and S. typhimurium using only two nanorods as a pathogen biosensor. The development of multiple labels for cancer diagnosis in vitro will have the further potential of such assay applications, as demonstrated by these studies [36].
A small number of studies have investigated the use of multiple plasmonic NPs simultaneously towards multiplex molecular imaging applications. A study by Yu et al. demonstrated darkfield imaging and spectroscopy for immunophenotype profiling of cells using three nanorods of increasing aspect ratio, resulting in scattering peaks within three distinct spectral windows [37]. Immunophenotype composition was determined from relative NP scattering intensity and found comparable to results using established flow cytometry techniques. A follow-on study again used nanorods of distinct scattering spectral windows labeled with HER-2 and EGFR antibodies to profile cancer cell lines through photoacoustic molecular imaging. In this study, only one individual label was exposed to each particular cell line [38]. Results were able to determine an immunophenotype with comparable performance to Western blot techniques. A later work by the same group next demonstrated triple labeling using HER-2, EGFR, and CXCR4 bound nanorods, but only implemented two conjugates at a time and within animal models to show delivery [39]. A third study was the first to multiplex using gold nanorod labels in combination with silver nanospheres. In this study, particles were conjugated to transferrin and anti-mesothelin. Both darkfield and TEM imaging were conducted on the labeled pancreatic cancer cells. Experiments also tested for cell viability with the different conjugates over 48 hours and found that the labeled cells maintained viability over 80% [40].
2.4.3 Cytotoxicity

Cytotoxicity concerns of these noble metal plasmonic NP labels have previously been investigated. For instance, gold nanospheres have been found to be taken up by cells. However, under short term exposure, gold NPs themselves do not cause acute cytotoxicity [41, 42]. Potential toxicity may arise from chemicals used in the fabrication and stabilization process. Gold nanorods exhibit similar biocompatibility behavior. While the rods themselves are biocompatible, the nature of the particles requires a surfactant to prevent aggregation. The commonly implemented chemical is cetyltrimethylammonium bromide (CTAB). Observed cytotoxicity is primarily attributed to free CTAB remaining in solution from the fabrication process [43]. CTAB that remains bound to the rods is found to be far less toxic. The full extent of biocompatibility of silver nanospheres remains to be seen, and requires further study. One investigation found the toxicity of silver nanospheres in embryos is dependent on the concentration of the particles, which affects rates of passive diffusion and resulting accumulation [44]. Nonetheless, surface modification treatments are being developed to improve the biocompatibility of all three of these markers [45].
2.5 **Targeted receptor tyrosine kinases**

2.5.1 **ErbB receptor family**

The ErbB receptor family consists of four structurally similar receptor tyrosine kinases, including epidermal growth factor receptor (EGFR/ErbB-1), human epidermal growth factor receptor 2 (HER-2/ErbB-2), ErbB-3, and ErbB-4. These receptors are responsible for the regulation of multiple signaling pathways that control cell proliferation, differentiation, survival, mobility, and adhesion [46, 47]. Upon growth factor ligand binding, these receptors become activated forming either homodimers or heterodimers, depending on several factors. For instance, the primary function of HER-2 appears to be as a heterodimeric partner for other members of the ErbB family because it does not undergo activation from growth factor ligands [47, 48]. However, when cells express high levels of HER-2 the receptor undergoes constitutive homodimerization [46, 49]. While over-expression of either EGFR or HER-2 can indicate increased metastatic potential, clinical trials have shown that the increased presence of both receptors simultaneously results in a more accurate indicator of degree of cancerous activity than either when expressed alone [50]. The ability to monitor the dimerization behavior of EGFR and HER-2, including the measurement of dimer spacing, may provide a better understanding of the mechanisms in which these different receptor signaling pathways interact.
2.5.2 IGF-1R

The insulin-like growth factor 1 receptor (IGF-1R) is a heterotetrameric transmembrane receptor tyrosine kinase that plays a role in oncogenic transformation. The receptor is commonly overexpressed in tumors, resulting in inhibition of apoptosis, the mechanism of programmed cell death. The receptor is comprised of two extracellular subunits of 135 kDa and two transmembrane beta subunits of 95 kDa, containing the intracellular tyrosine kinase [51]. IGF-1R is overexpressed in several tumor types including melanoma, colon, and pancreas [52]. A positive correlation has also been found between the level of IGF-1R expression and favorable prognosis in breast cancer [53].

Ligand binding partners include insulin-like growth factor 1 and insulin-like growth factor 2, with high and low affinities, respectively. Upon binding the tyrosine kinase phosphorylates insulin receptor substrate 1 (IRS-1) and SHC (SRC homology protein), these proteins form a complex with GRB2, SHC-2, and SHC-3. The complex then activates both the Ras/Raf/mitogen activated protein kinase (MAPK) phosphatidylinositol 3-kinase (PI 3K)-Akt pathways [51, 54]. These signal pathways result in cellular transformation, apoptosis inhibition, and motility [54].
2.5.3 Receptor Interactions

2.5.3.1 EGFR and HER-2

Co-expression of EGFR and HER-2 can result in heterodimers, causing transphosphorylation and activation of three major signaling pathways [55], including: the PI3K-Akt pathway which leads to phosphorylation of pro-apoptotic Bad by Akt, blocking interaction with Bcl-2 and Bcl-X, and leading to evasion of apoptosis; the PLC-PKC pathway which leads to cellular motility; and the RAS-MAPK pathway which leads to phosphorylation of MAPK and subsequent phosphorylation and activation of multiple transcription factors leading to cell cycle progression.

The crosstalk between the two receptors has direct implications for therapy. One drug therapy in early clinical trials directly targets the interaction between these two receptors as they dimerize. Pertuzumab blocks HER-2/EGFR dimer signaling upon ligand binding through steric inhibition [56, 57]. A second drug, the anti-estrogen tamoxifen, is even more effective in the treatment of breast cancer. However, cells can acquire tamoxifen resistance through an increase in EGFR/HER-2 dimerization and signaling activity [58].

2.5.3.2 EGFR and IGF-1R

Co-expression of EGFR and IGF-1R commonly results in formation of heterodimers between the receptors [59]. Upon IGF-1 ligand interaction with the
receptors, the ERK pathway becomes activated, resulting in cell proliferation.

Transactivation of the two receptors also can result in an increase in PI3K/Akt activity [59]. EGFR actually increases IGF-1R protein stability through complex formation [60]. This complex activates ERKs upon IGF-1 binding [61]. The signal pathways of EGFR and IGF-1R also present overlapping downstream signaling, which may be due to src PTK or IGF-binding protein-3, both of which may regulate cross talk between the receptors [59, 62]. EGFR can also inhibit the IGF-1R signal pathway through the IRS-1 adaptor protein [59]. Additional evidence suggests IGF-1R induced phosphorylation of EGFR through metalloprotease-dependent release of EGF-like ligands [61].

Again, the cross-talk between these two receptors has direct implications for potential therapies. One particular drug therapy, gefitinib, targets the crosstalk between EGFR and IGF-1R through inhibition of EGFR [61]. Evidence suggests the IGF-2/IGF-1R survival pathway confers resistance to gefitinib treatment [61], and that there appears to be an increase in IGF-1R signaling during gefitinib treatment [59]. For these reasons, co-targeting of both receptors may increase treatment success.

2.5.3.3 IGF-1R and HER-2

The interactions between IGF-1R and HER-2 are minimal in most cells. Nonetheless, IGF-1R signaling can cause phosphorylation of HER-2 [59]. Interesting interactions arise in trastuzumab resistant cells. Trastuzumab, a monoclonal antibody,
targets HER-2 and is commonly used in treatment of HER-2 positive breast cancers. In these resistant cells, IGF-1R will form heterodimers with HER-2. [63]. In addition, under these conditions IGF-1 will also cause phosphorylation of HER-2. For these reasons, the use of anti-IGF-1R agents as part of a co-targeting strategy restores sensitivity to trastuzumab [59].

As discussed above, cancer therapies that selectively target only one step in a very complex signaling pathway often encounter problems. When posed with an interruption in one pathway, these cells often adapt to utilize alternative mechanisms to maintain growth. Therefore, it makes sense to target therapeutics against multiple pathways simultaneously to reduce the available signaling mechanisms. The ability to monitor the activity of all three receptors simultaneously with plasmonic NPs would provide an additional tool in the development of these new therapies.

### 2.6 Basics of flow cytometry

Flow cytometry provides quick and easy analysis of large populations of cells, including measurements of size, granularity, and fluorescence intensity [64]. The basics of flow cytometry involve laser illumination of a stream of particles, particularly cells. Depending on the application, cells are usually labeled with a fluorescent tag of a specific membrane receptor and then fixed in either formaldehyde or left in a live cell buffer. A fluidics system then carries the cell suspension towards a flow chamber where
it is injected into a stream of sheath fluid. Sheath fluid pressure results in hydrodynamic focusing, which causes the cells of the suspension sample core to align in the center of the stream in a single file line. At this point the cells pass through the incident laser beam. Depending on the cell properties, the light scatters in multiple directions to different extents. Forward scattered light is proportional to the cell size. Side scattered light is proportional to cell granularity and internal cellular structures. Incident light also excites any fluorophore tags bound to the cell membrane. The emitted fluorescence is collected and delivered to multiple filter channels specific to individual fluorescent tags. The intensity recorded corresponds to a relative expression level of the receptor of interest.

The flow cell is also capable of facilitating fluorescence activated cell sorting (FACS). The sample stream is vibrated at a frequency that breaks up the suspension into droplets containing individual cells, each at a set distance apart. The flow cytometer user determines the ideal cell properties for the discrimination. When these properties are met, the system applies a charge to that particular cell’s droplet, differentiating it from the rest of the population. As the droplet passes through the electric field of a charged plate, it deflects the cells of interest into a collection tube.
3 Development of hyperspectral imaging system

In this chapter, a hyperspectral imaging system based around the current darkfield epi-illumination microscope is implemented, tested, and validated. Hyperspectral imaging will allow for a continuous spectrum to be recorded at each spatial point in the field of view and easy spectral profiling of individual NP labels. The previous system iteration was limited, requiring multiple spectral line acquisitions across cells of interest to adequately characterize receptor expression. The system will also provide the ability to excite and record from cells at specific wavelengths corresponding to the peak resonance of the various NP labels, allowing for simple immunophenotype profiling applications. Validation of the system will be conducted through comparison of gold nanosphere scattering to theoretical values predicted through Mie theory.

3.1 System

The original darkfield microspectroscopy system consisted of an inverted microscope (Axiovert 200, Zeiss), a line imaging spectrometer (SpectraPro 2150i, Acton Research) containing an imaging CCD (Spec-10, Roper Scientific), and a color camera (CoolSnap cf, Photometrics, Tucson, AZ). The system utilizes a custom designed epi-illumination darkfield light train [65], rather than transmitted illumination, which has the advantage of eliminating forward-scattered light from cellular structures which can
mask NP-scattered light in transmission darkfield experiments. The resulting enhancement in contrast allows smaller concentrations of NPs to be used and identification of small pockets of receptor expression. The system also allows for concurrent bright field imaging which makes it easier to observe the dimensions of the cell itself in addition to areas of receptor expression.

This novel darkfield microscopy configuration has advanced NP characterization and demonstrated the use of new immunolabeled NP tags. Despite the benefits of the current system however, certain limitations do exist that can be addressed. Primarily, conventional spectral analysis acquired with a line imaging spectrometer does not adequately relate spatial and spectral information. Furthermore, the acquisition of multiple spectral lines does not sufficiently represent all objects in the field of view. In fact, conducting spectroscopy experiments in this manner significantly limits overall throughput. Cellular receptor binding experiments requiring large sample sizes only exasperates the problem.

Because of these limitations, the darkfield microspectroscopy system was adapted to incorporate hyperspectral imaging capabilities (Figure 3.1). A broadband source coupled to an acousto-optic tunable filter provides narrow band illumination to the current epi-illumination setup. An image is taken at each wavelength, creating a hyperspectral data cube with two spatial and one spectral dimensions. The improved configuration offers several benefits. Foremost, a continuous spectrum can be recorded
at each spatial point within the field of view. Additionally, the widely tunable AOTF provides excitation over a broad range of visible wavelengths. Small spectral intervals of illumination can thus be chosen allowing for subtle differences in scattering behavior from spectrally distinct NPs to be better characterized. Finally, the increased speed provided by a high frame rate camera allows a total recording time of the hyperspectral data cube on the order of seconds, representing a significant improvement in overall acquisition time.

Figure 3.1: Hyperspectral darkfield microscopy system for analysis of live cells in culture. The system is comprised of a Zeiss inverted microscope, Cascade:650 imaging CCD, Fianium SC450-2 supercontinuum light source, and Crystal Technologies acousto-optic tunable filter.

3.1.1 Light source

The implemented broadband source is a Fianium SC450 supercontinuum source [66]. This high power fiber laser (Class 4) provides up to 2 W broadband with over 1.5 mW/nm (Figure 3.2). The spectral range covers 450 to 2500 nm with pulse to pulse noise at center wavelength 1064 nm <1%, although it increases to 4% into the red, and 10% into
the blue. The master source consists of a passively mode-locked low power fiber laser containing Yb-doped gain media. The 20 MHz repetition rate translates into 6 ps output pulses. A laser diode pumps the doped fiber amplifier. The double clad Yb-doped fiber amplifies the multiplexed signal through stimulated emission of photons from the dopant ions. A highly non-linear fiber causes spectral broadening, generating a supercontinuum. A collimating lens at the end of the optical fiber results in a beam divergence between 0.01-0.03 rad, depending on wavelength.

![Spectral profile of supercontinuum source at increasing power levels](image.png)

*Figure 3.2: Spectral profile of supercontinuum source at increasing power levels [66].*

### 3.1.2 Acousto-optic tunable filter

An acousto-optic tunable filter consists of a piezoelectric transducer bound to a birefringent lithium niobate crystal. The transducer results in acoustic compression waves traveling through the crystal creating a Bragg diffraction grating. At different frequencies of modulation the crystal undergoes different periods of RI variation
causing different wavelengths to be diffracted. The filter then isolates the first order diffraction beam.

While mechanical filter wheels provide an alternative method for wavelength selection, they suffer from several limitations. Foremost, hyperspectral applications require specification of over 50 wavelengths. Depending on the observed sample, different wavelength ranges at varying increments may be more appropriate. Filter wheels do not provide enough flexibility for such adaptation. Additionally, over time bandpass filters suffer from heat deterioration, unlike AOTF crystals. Finally, a rotating filter wheel introduces mechanical complications entirely avoided when using an AOTF.

A Crystal Technologies acousto-optic tunable filter is coupled to the broadband source fiber [66]. This widely tunable electro optic device provides operating wavelengths from 450 to 700 nm with a manufacturer specified average spectral bandwidth of 3.5 nm at 515 nm. Eight channels are available, capable of 5 mW of power for each, although only one is necessary for this hyperspectral application. The filter does not significantly change the beam divergence output from the supercontinuum source. Under a source of linear polarization, the effective transmission of the filter can be as high as 85%, but due to the random polarization of the broadband source used, the effective transmission is actually 45%. Available drivers allow easy control through Labview. AOTF output is coupled into a 200 µm multi-mode fiber using an f=7.5 mm collimating visible achromat.
3.1.3 Camera

The Cascade 650 CCD (Photometrics, Tucson, AZ) provides many features necessary for a hyperspectral imaging application. First, the camera can achieve 10 MHz high speed acquisition. This high frame rate translates to 27 fps at the full field of view or 100 fps at a smaller 128 x 128 region of interest. Hyperspectral imaging requires such frame rates to adequately image dynamic cell processes. Second, the 653 x 492 imaging array contains pixels measuring 7.4 x 7.4 µm. The size is ideally matched to an optical microscope providing diffraction-limited resolution of fine details. Third, on-chip multiplication gain and relatively high quantum efficiency achieve high sensitivity. The quantum efficiency is over 35% for the spectral range of the AOTF of 450-700 nm, although it is actually over 50% for wavelengths between 500-800 nm. 16-bit digitization accurately detects this dynamic range of signal intensity. Fourth, the camera is compatible with Labview drivers, allowing for easy integration with the AOTF. Other important camera characteristics include thermoelectric cooling down to -35°C, linear full well of 27,000 e-, read noise of 25 e- rms, and dark current of 1 e-/p/s.

3.2 Characterization

While the AOTF is capable of up to 5 mW of power per wavelength channel, at these larger powers harmonic content in the RF amp causes broadening of the output spectral width. Lower AOTF output powers of 1.0 mW minimize this effect. On average,
the lower output results in a measured FWHM of 8.5 nm over the 450-700 nm spectral range. Figure 3.3 demonstrates that the bandwidth increases over longer filtered wavelengths.

![Output Spectra of AOTF](image)

**Figure 3.3: Spectra profile of AOTF output at various wavelength intervals.**

Across the visible spectrum the supercontinuum source does not output a constant power. The AOTF power is directly proportional to the source and therefore requires correction to maintain even power for each wavelength increment. The calibration procedure involves using a power meter to determine the power setting for each wavelength, thus providing constant power output.

The high frame rate of the camera should allow up to 27 fps for the full 653 x 492 imaging array. At 40x magnification, observed cells rarely exceed a 170 x 170 region of interest. Such a small size should translate into nearly 100 fps, assuming a 0 s integration time. In practice, the actual acquisition speed is not integration-time limited due to camera driver constraints when triggering from a corresponding AOTF wavelength.
increment. A 170 x 170 pixel region of interest acquired from 450 to 700 nm with 5 nm (51 images with 30 ms integration times) and 1 nm (251 images with 30 ms integration time) increments takes ~2.4 s and ~12.0 s, respectively, for acquisition. While these speeds are sufficient for cell imaging, observation of more dynamic processes would require further improvement.

The supercontinuum source, even after calibration, will have wavelength dependent variations. Source-correction is performed by normalizing the signal by source intensity, as measured using a diffuse reflectance standard (WS-1, Ocean Optics, Dunedin, FL). A reference image is acquired to perform background subtraction. A dark reference is also acquired and subtracted from all signals. The following equation represents the final processed scattering spectrum:

\[ I_{\text{final}} = \frac{(I_{\text{ROI}}-I_{\text{dark}})}{(I_{\text{source}}-I_{\text{dark}})} - \frac{(I_{\text{bkgd}}-I_{\text{dark}})}{(I_{\text{source}}-I_{\text{dark}})} \]  

[3.1]

where \( I_{\text{final}} \) is the true scattering intensity of the sample, \( I_{\text{ROI}} \) is the measured scattering intensity of the sample, \( I_{\text{bkgd}} \) is the background scattering intensity, \( I_{\text{dark}} \) is the dark measurement of the CCD, and \( I_{\text{source}} \) is the reference source intensity. Wavelength-dependent effects of the CCD are also removed through source normalization. The following equation demonstrates how this is possible:

\[ \frac{I(x,\lambda)_{\text{NP}}}{I(x,\lambda)_{\text{source}}} = \frac{S(x,\lambda)\cdot P(\lambda)\cdot Q(\lambda)\cdot \eta(\lambda)}{1\cdot P(\lambda)\cdot Q(\lambda)\cdot \eta(\lambda)} = S(x,\lambda) \]  

[3.2]
where $I_{NP}$ is the measured intensity of the NPs, $I_{source}$ is the measured reference source intensity, $S$ is the scattering intensity of the NPs, $P$ is the power spectral density, $Q$ is the electric charge, and $\eta$ is the quantum efficiency.

### 3.3 Validation

The updated hyperspectral configuration is now capable of acquiring a continuous spectrum at each spatial point within the field of view. The imaging system still requires testing and validation. One validation approach takes advantage of the scattering profile of gold nanospheres. Mie theory can accurately predict the extinction spectra of these spheres. Therefore, the NPs are bound to silanated glass substrates and immersed in several media of varying refractive indices. The hyperspectral profiles of the NPs are then compared to the Mie theory predictions.

Glass substrates are prepared by cleaning glass coverslips with RBS detergent at 80° C under sonication for ten minutes. Washing is followed by a water rinse. Coverslips are then cleaned in a methanol/hydrochloric acid mix (1:1) again under sonication for 30 minutes. Washing is followed by a water rinse and then a subsequent ethanol rinse. Coverslips are then dried by oven at 60° C overnight. Slides are incubated in a 10% MPTES solution in ethanol for 15 minutes. A thiol-terminated silane self-assembled monolayer (SAM) forms on the surface on the slide. Incubation is followed by a set of
five rinses in ethanol under sonication for one minute each. The slides are finally dried for three hours in an oven at 120° C [67].

Validation experiments compared the spectra of the same 80 nm gold NPs bound to the silanated glass slides in water, oil, and increasing concentrations of glycerol. The refractive indices of each immersion media are measured using a refractometer (RFM 340, Bellingham Stanley, Kent, UK) were found to be 1.33 for water, 1.36 for 20% glycerol, 1.39 for 40% glycerol, 1.42 for 60% glycerol, 1.45 for 80% glycerol, and 1.52 for immersion oil. To account for the contributions from both the immersion media and the glass substrate, the effective RI was calculated according to the following equation with a weighting factor $\alpha$ of 0.58 and borosilicate substrate RI $n_{substrate}$ of 1.52 [68, 69].

$$n_{eff} = \alpha \cdot n_{medium} + (1 - \alpha) \cdot n_{substrate}$$

[3.3]

Mie theory calculations were performed in MiePlot [70] using these effective RI values and optical constants for gold experimentally determined by Johnson and Christy [71]. Both experimental and theoretical spectral profiles were then fit to Gaussian peaks to determine peak scattering.

The hyperspectral system acquired peaks from N=8 NPs in each of six immersion media (Figure 3.4). Peak scattering was experimentally determined to be 571.0 ± 3.2 nm for water, 576.2 ± 4.3 nm for 20% glycerol, 578.5 ± 5.1 nm for 40% glycerol, 581.3 ± 5.9 nm for 60% glycerol, 585.0 ± 6.1 nm for 80% glycerol, and 592.0 ± 5.8 nm for immersion oil (Figure 3.5). Mie theory calculations determined peak values of 572.2 nm for water, 574.4
± 0.03 nm for 20% glycerol, 577.1 ± 0.03 nm for 40% glycerol, 579.6 ± 0.03 nm for 60% glycerol, 582.1 ± 0.03 nm for 80% glycerol, and 588.3 ± 0.03 nm for immersion oil (Figure 3.5). Considering that a spectral resolution of 5 nm was used to acquire this data, the experimental data was close in agreement (Figure 3.5). Deviations from Mie theory may be attributed to an actual distribution greater than the manufacturer-specified 60 nm size, and slight peak broadening of experimental data due to the bandwidth of the AOTF output.

Figure 3.4: Hyperspectral image of 60 nm gold nanospheres immersed in water. Images are converted to color using a CIE 1964 10° Standard Observer RGB filter [72].

Figure 3.5: Experimental (left) and theoretical (right) scattering spectra of 80 nm gold nanospheres in water and 80% glycerol.
Figure 3.6: Comparison of peak resonant wavelengths for immersion media with increasing refractive indices (Theoretical $R^2 = 0.9993$, Experimental $R^2 = 0.9921$).
4 Comparison of nanoparticle and fluorescence based receptor quantification

In this chapter, an assessment is performed comparing immunolabeled NP biomarkers to fluorescent marker imaging for the measurement of epidermal growth factor receptor expression [73]. Fluorescent techniques remain the gold standard for quantifying receptor levels, but immunolabeled NPs reveal additional information about their local nanoenvironment via RI sensitivity. Furthermore, unlike fluorescence, NPs are biocompatible and not susceptible to photo-bleaching.

In the present study, molecular imaging using immunolabeled NPs is compared to fluorescent marker imaging for characterization of tumor cells across four cell lines. An A431 human epidermoid carcinoma cell, which is notable for high EGFR expression, is utilized. Two cell lines, 270-GBM human glioblastoma xenograft cells and H2224 human glioblastoma xenograft cells, which have lower EGFR expression, are also employed. Finally, a fourth cell line, MDA-MB-453 breast carcinoma cells, was known to not express EGFR. The hypothesis is that (1) immunolabeled NPs would function at least as well as fluorescent marker imaging for measuring receptor expression and (2) still provide additional information descriptive of the specific nanoenvironment. Our hypothesis would be verified if the following conditions were met: (1) if a linear relationship was shown between the intensity of light scattered by immunolabeled NPs bound to cell surface EGFR and the fluorescence intensity using a conventional marker
and (2) if differences between cell lines in the peak wavelength of the scattering spectra could be seen, reflecting different responses by NPs to the local nanoenvironment.

4.1 Materials and methods

Microspectroscopy system

Experiments were performed using the original darkfield microspectroscopy system described in Section 3.1, prior to the hyperspectral additions.

Preparation of Cell Cultures for the Four Cell Lines

A431 human epidermoid carcinoma cells were incubated at 37° C and 5% CO₂ using Dulbecco’s Modified Eagle’s Medium (DMEM), with 10% fetal bovine serum and 1% penicillin streptomycin. 270-GBM human glioblastoma xenograft cells were incubated at 37° C and 5% CO₂ using Zinc Option Medium, with 10% fetal bovine serum and 1% penicillin streptomycin. EGFR non-expressing MDA-MB-453 breast carcinoma cells were incubated at 37° C using Leibovitz L-15 Medium, with 10% fetal bovine serum and 1% penicillin streptomycin. FACS selected low EGFR-expressing H2224 human glioblastoma xenograft cells were incubated at 37° C and 5% CO₂ using Modified Eagle’s Medium (MEM) / F12 Stem Cell Medium, with 1% gluta-max I, 1% non-essential amino acids, and 1% penicillin streptomycin.
For both fluorescence and NP experiments, 80k cells were plated in 2.0 mL chambered cover glasses (155380, Nunc, Rochester, NY) in 1 mL of media. Cells were incubated overnight for NP experiments and an additional 24 hours for fluorescence experiments. All split cells were kept within 3 passages to maintain EGFR expression levels.

**Antibody-NP conjugation**

Immunolabeled NPs (i.e., antibody-NP conjugates) were prepared with 0.5 mL of 60 nm diameter spherical gold colloid with no distinct surface coating at a concentration of $2.6 \times 10^{10}$ particles/mL (15709-20, Ted Pella, Inc., Redding, CA) and 15 µL anti-EGFR antibodies (E2156, clone 225, Sigma-Aldrich, St. Louis, MO) at a concentration of 1.5 mg/mL. The gold colloid was diluted in 485 µL of 20mM HEPES buffer and the anti-EGFR antibody was diluted in 62.5 µL of 20mM HEPES buffer. The pH of each solution was adjusted to 7.0 ± 0.2 using 100 nM K$_2$CO$_3$. The pH-adjusted solutions were then mixed using an oscillator at 190 cycles/min for 20 minutes. 100 µL of the solution was removed and mixed with 5 µL of 10% NaCl to test for antibody-NP conjugation. A resulting change in color indicates incomplete antibody coverage of the NPs, which causes them to aggregate. 100 µL of 1% PEG (P2263, Sigma-Aldrich, St. Louis, MO) was then added to the conjugate solution, coating areas of the NPs not bound to the antibodies. After 10 minutes of interaction, excess PEG was removed by centrifuging the
solution for 15 minutes at 6000 RPM. After removal of the supernatant, the NP pellet was resuspended with 0.5 mL of 1x phosphate buffered saline to yield a final NP concentration of $2.35 \times 10^{10}$ particles/mL.

**Cell treatment with NPs**

Split cells were plated for NP and fluorescence experiments simultaneously. The NP-designated cells were incubated overnight. Then, the media was removed and replaced with a solution of 0.55 mL media and 0.45 mL NP suspension. Cells were then incubated for an additional 20 minutes, allowing interaction with the immunolabeled NPs. Upon removing the NP media solution, cells were washed twice with fresh media and then replaced once more. To ensure the viability of cell samples after washing, the media was warmed to $37^\circ$ C prior to the wash and a gentle hand pipette technique was used rather than an automatic pipette. Experiments on the microspectroscopy system were conducted immediately afterwards.

**Nanoparticle spectral measurement**

The microspectroscopy system was used for NP-scattering measurements of cell samples. A heated stage maintained the chambers at $37^\circ$ C during measurements. Cells that exhibited observable conjugate binding were randomly chosen for analysis. A spectral line with a spatial width of 2.0 µm was moved in regular intervals of 3.0 µm.
across cells chosen for analysis. At each interval, spectra were collected using a 20 s exposure time. In addition, a corresponding color image was collected for spatial identification of the location of the spectral data.

Pure gold colloid and unbound NP conjugates were analyzed using transmission experiments with a quartz tungsten halogen (QTH) light source (LS-1, Ocean Optics, Dunedin, FL) and a compact linear CCD spectrometer (USB2000, Ocean Optics, Dunedin, FL).

*Fluorescence imaging*

Fluorescence experiments were conducted using mouse monoclonal IgG1 Anti-EGF Receptor Alexa Fluor 488 Conjugate (16-246, clone LA1, Millipore, Billerica, MA) as a contrast agent. 2 mL chambered cover glasses were plated with 80K cells at the same time as cells designated for NP experiments, but were incubated for an additional 24 hours. Cell media was exchanged for a dilution containing 8 µL of the conjugate and 1 mL of cell media. Cells were then incubated for an additional 10 minutes, allowing the conjugate to interact with the cells in darkness. Afterwards, the dilution was removed, washed with 1x phosphate buffer solution, and then given 1 mL fresh media. Fluorescence experiments were immediately conducted using an inverted microscope (Axiovert 200, Zeiss, Oberkochen, Germany) equipped with a Xenon arc lamp source, DAPI fluorescent filter, and color CCD camera (CoolSnap cf, Photometrics, Tucson, AZ).
Images were collected over a period of one hour using exposure times of 0.3 s. Cells that exhibited observable conjugate binding were randomly chosen for analysis.

**Western blot assay**

For Western blot assays [74], 80% confluent cells were washed two times with PBS and scraped into M-PER Mammalian Protein Extraction Reagent (78503, Thermo Fisher Scientific, Waltham, MA). Cells were lysed 15 minutes on ice and insoluble materials were removed by centrifugation. Total protein concentrations in cell lysates were determined with a BCA Protein Assay kit (Bio-Rad, Hercules, CA). 50 µg samples were separated by SDS–PAGE and electro-transferred to a PVDF membrane. After overnight blocking with block buffer at 4°C, the membrane was incubated one hour with rabbit anti-EGFR antibody (1:500; 2232, Cell Signaling Technology, Danvers, MA) and then rinsed three times for 15 minutes each by TBST. The membrane was then incubated in anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000) for one hour, rinsed six times for 15 minutes each by TBST, and exposed to chemi-luminescence using ECL kits (NA934V, GE Healthcare, Chalfont St Giles, UK). Internal control GAPDH was also detected in parallel with a polyclonal anti-GAPDH antibody (sc-25778, Santa Cruz Biotechnology, Santa Cruz, CA).
Data Analysis

In order to compare data across cell lines, the NP-scattering and fluorescence intensities were normalized to cell area, which ensured that larger cells did not present higher expression levels due to their increased area. To compare data for the two imaging modalities, normalized intensity values were obtained for both NP and fluorescence experiments. For NP-scattering data, intensity was determined by measuring the spectral peak area for each cell interval. Peaks were isolated by subtracting background spectra associated with cellular structure scattering (Figure 4.1). This baseline signal was represented by a best fit to a simple power law model based on the observed trend. The peak areas were then summed for each cell and normalized to the total area of the cell. For fluorescence data, intensity was determined to be the mean pixel intensity for the entire cell using ImageJ software (NIH). The peak wavelengths of the NP-scattering spectra were determined by fitting a Gaussian function to each spectrum and locating the center wavelength.
Figure 4.1: Graphs showing scattering intensity from cells bound to NPs compared with isolated background cellular scattering for each of four cell lines examined in this study. A) A431 cells, which are known to have high EGFR expression. B) 270-GBM cells, which have moderate EGFR expression. C) H2224 cells, which have lower EGFR expression. D) MDA-MB-453 cells, which do not express EGFR.

**Statistical Analysis**

To account for distributions that did not necessarily adhere to a normal distribution, all $p$-values were calculated by using a Wilcoxon rank sum test in Matlab (Mathworks, Natick, MA) using the default value of $\alpha = 0.05$. The $R^2$ value for the relationship between NP-scattering and fluorescent intensity was established using a linear regression in Matlab.
4.2 Results

Darkfield images of the same EGFR-expressing cell line showed a clear scattering-intensity difference between cells not exposed to NPs and cells bound to NPs (Figure 4.2). NP-scattering from NP-bound cells showed a significant increase in intensity over cells with no NP exposure, which presented only low inherent scattering primarily from cellular structures.

Figure 4.2: Epi-illumination images of EGFR-expressing A431 cells. Image shows EGFR-expressing A431 cell that was bound with immunolabeled NPs (left), with green regions representing sites of NP-binding at receptors. Compare NP light-scattering of this cell with that of the EGFR-expressing, A431 cell, that were not exposed to any NPs (right), in which no light-scattering is seen. (Scale bars = 20 μm)

A431 human epidermoid carcinoma cells

A431 cells (N= 150) were examined using both imaging modalities. They presented 35.3 ± 0.65 (SEM) fluorescence counts per cell with a standard deviation of 8.1 counts. The average NP-scattering for these cells was found to be 41.5 ± 1.7 intensity counts per cell with a standard deviation of 20.1. From the spectral data, the average
peak wavelength of the scattering was determined to be $570.93 \pm 1.42$ nm with a standard deviation of 17.42 nm.

**270-GBM human glioblastoma xenograft cells**

270-GBM cells (N= 147) were examined using both imaging modalities. They presented $28.7 \pm 0.89$ fluorescence counts per cell with a standard deviation of 10.8 counts. The average NP-scattering for these cells was found to be $24.7 \pm 2.0$ intensity counts per cell with a standard deviation of 14.8. From the spectral data, the average peak wavelength of the scattering was determined to be $565.26 \pm 1.53$ nm with a standard deviation of 18.72 nm.

**H2224 human glioblastoma xenograft cells**

H2224 cells (N= 40) were examined using both imaging modalities. A fewer number of cells of this type were examined as the cell culture exhibited sparse growth. The examined cells presented $5.91 \pm 0.20$ fluorescence counts per cell with a standard deviation of 2.5 counts. The average NP-scattering for these cells was found to be $8.77 \pm 1.0$ intensity counts per cell with a standard deviation of 6.5. From the spectral data, the average peak wavelength of the scattering was determined to be $562.70 \pm 3.84$ nm with a standard deviation of 25.60 nm.
**MDA-MB-453 breast carcinoma cells**

MDA-MB 453 cells (N= 50) were examined using both imaging modalities. Here the negative binding was so uniform that the need to examine more cells was obviated. The examined cells presented 2.07 ± 0.10 fluorescence counts per cell with a standard deviation of 0.68 counts. The average NP-scattering for these cells was found to be 0.44 ± 0.11 intensity counts per cell with a standard deviation of 0.77. No clear peaks were observed in the spectral data so no meaningful average peak wavelength data were obtained.

A431 and MDA-MB-453 cell lines were obtained from the ATCC through the Duke Cell Culture Facility. 270-GBM and H2224 cells were provided by the lab of Dr. Gerald Grant.
4.2.1 Comparison across cell lines

![Cell Images](image)

Figure 4.3: Darkfield epi-illumination NP images (upper row) and fluorescence images (bottom row) of an A431 cell (left), a 270-GBM cell (middle left), and an H2224 cell (middle right), which express EGFR at different levels. For comparison, a non-EGFR-expressing MDA-MB-453 cell (right) is also shown. (Scale bar s = 20 µm)

Qualitative examination of darkfield images (Figure 4.3) revealed that upon tagging with immunolabeled NPs, the EGFR-expressing A431 cells exhibit increased scattering compared to the 270-GBM and H2224 cells. In contrast, the EGFR non-expressing MDA-MB-453 cells showed minimal amounts of scattering. The increased intensity of NP-scattering provided adequate contrast for distinguishing receptor-expressing from non-receptor-expressing cells, while also remaining low enough to maintain observations of individual receptor binding events. Non-receptor-expressing cells had low scattering comparable to unlabeled receptor-expressing cells, matching our previous results using a similar experimental scheme [16]. Fluorescence images further validated the similar intensity differences across the cell.
A Western blot assay (Figure 4.4) confirmed the qualitative EGFR expression differences among the A431, 270-GBM, and MDA-MB-453 cells. The Western blot assay used an antibody that bound both wild-type EGFR and EGFR VIII mutant. Although these variants are discriminated by molecular weight in the assay, the antibody used in the NP experiments bound to both. Thus, to compare across cell lines it is necessary to combine the contributions from both variants in the Western blots. On combining the contributions from both, the A431 cells have a high EGFR expression level, the 270-GBM cells have a comparable but lower level, and the MDA-MB-453 cells have no appreciable expression.
Figure 4.5: Mean normalized fluorescence intensity and standard error of measurement of four different cell lines including A431 (N=150), 270-GBM (N=147), H2224 (N=25), and MDA-MB-453 (N=40). **p < 0.0001 between A431 and 270-GBM cell lines and also between 270-GBM and H2224 cell lines. Mean normalized NP scattering intensity and standard error of measurement of four different cell lines including A431 (N=150), 270-GBM (N=147), H2224 (N=40), and MDA-MB-453 (N=50). **p < 0.0001 between A431 and 270-GBM cell lines and also between 270-GBM and H2224 cell lines.

For a quantitative comparison, the data for the average fluorescence and NP-scattering intensities are summarized graphically in Figure 4.5. For both modalities, the different cell lines presented statistically significant (p<0.0001) differences in intensity levels when compared within the technique.

4.2.2 Comparison across techniques

To compare across the techniques, widefield images of multiple 270-GBM cells are presented in Figure 4.6. Both the NP scattering and fluorescence intensity are consistent from cell to cell. For a quantitative comparison, a plot of the fluorescence
intensity versus NP-scattering intensity is shown in Figure 4.7. The trend line presents the best fit obtained using linear regression. The data are seen to agree with a linear relationship given by $\text{NP} = 1.0691 \times \text{FL} - 0.3873$, where NP is the intensity of the NP-scattering and FL is the fluorescence intensity. The covariance of the data with the trend line is $R^2 = 0.9409$. Overall, these results demonstrate that immunolabeled NPs were as effective as fluorescent markers in quantifying receptor expression.

![Image](image1.png)

**Figure 4.6: Comparison of molecular imaging of multiple 270-GBM cells using both imaging techniques.** (left) Image shows examples of several cells bound with NPs immunolabeled against EGFR under darkfield illumination. (right) Image shows examples of several cells bound with fluorescent markers for EGFR. (Scale bar = 20 µm)

In addition to analysis of the NP-scattering intensity, examination of the peak wavelength of NP-scattering revealed additional information about the cells (Figure 4.8). The A431 cells exhibited a peak scattering wavelength of $570.93 \pm 1.42$ nm with a standard deviation of $17.42$ nm, with the histogram of the data shown in Figure 4.8 (left). The 270-GBM cells exhibited a peak scattering wavelength of $565.26 \pm 1.53$ nm with a standard deviation of $18.72$ nm, with the histogram of the data shown in Figure 4.8.
(right). There was a statistically significant difference between these spectral data, with a p-value < 0.01. The H2224 cells (histogram not shown) exhibited a distribution of peak wavelengths that was not statistically different from either of the other cell lines while the MDA-MB-453 cells, due to the small amount of scattering, did not yield scattering data with peaks that were clear enough for analysis.

![Figure 4.7](image)

**Figure 4.7:** Plot of normalized NP scattering intensity and normalized fluorescence intensity for all four cell lines demonstrating linear relationship. ($R^2 = 0.9409$)

### 4.2.3 Discussion

EGFR expression levels have been shown to correspond with disease progression in certain tumor types [75-77]. Therefore, the ability to quantify receptor expression could potentially provide valuable clinical information, such as distinction of premalignant disease states from malignant ones, identification of dedifferentiation of low-grade tumors into more aggressive high-grade tumors, and monitoring of therapeutic response. A light-scattering technique is described using immunolabeled
NPs that quantitatively measures EGFR expression in vitro. This technique has advantages over the current reference standard, a fluorescent marker based method of measurement, including increased biocompatibility, better optical signal stability over time, and the possibility of optimizing optical properties for specific clinical applications. Furthermore, the potential utility of NP-scattering spectra as a means of distinguishing between cell types has been shown. The NP-scattering spectra exhibit changes in peak wavelength with local RI through the shift in plasmon resonance (Figure 4.8). This sensitivity at the nanoscale level provides additional functionalities of immunolabeled NPs that may allow for better characterization of tumor cells. The potential advantages of this potential functionality are discussed below in more detail.

In the microspectroscopy system used for this study, the standard darkfield configuration is modified to include an epi-illumination scheme [65]. This modification provided the advantage of avoiding detection of forward-scattered light from cellular organelles, resulting in high contrast between NPs and cells. The improved signal contrast allowed for administration of a smaller amount of NPs, which prevents saturation of membrane receptors by the immunolabeled NPs and instead enables highly sensitive detection of isolated NP binding events. Without the epi-illumination configuration, a substantial increase in NP concentration would be required to obtain adequate contrast using darkfield microscopy [17]. Because receptors are closely spaced on cell membranes (e.g., typically ~10 nm apart for EGFR [78]), application of a large NP
concentration for labeling would inevitably cause NPs to aggregate. Such an NP aggregation would be undesirable because the resulting plasmonic coupling causes a red shift in NP-scattering spectra that is no longer specific to the interaction of the NP with the nanoenvironment. This effect actually prevents detection of individual NPs and limits the ability to detect discrete sites of NP binding. In a previous study [16], it was shown through transmission electron microscopy (TEM) imaging that at the concentrations used herein, NPs taken up by A431 cells by the EGFR mechanism remain isolated in individual vesicles but tend to aggregate when taken up by non-specific means.

Fluorescent markers generally express a linear relationship between fluorescent intensity and target receptor expression [79]. In this experiment a linear relationship between NP-scattering intensity and fluorescence intensity is demonstrated by obtaining

![Figure 4.8: Histograms comparing peak NP scattering wavelengths in the A431 cell line and the 270 GBM cell line.](image)
a linear fit across the different cell lines with an $R^2$ value of 0.9409 (Figure 4.7). Thus, NP-scattering intensity highly correlates with receptor expression. It is noted that different antibody clones were used in the NP and fluorescent conjugates and that better agreement may be obtained upon using the same clones for both modalities.

Our findings of a linear relationship between the two techniques (Figure 4.7) contradict those of a previous study that found a quadratic relationship between NP-scattering and fluorescence intensity [17]. The difference in results can be attributed to three differences in experimental design. First, the previous study used transmitted illumination rather than our epi-illumination technique and thus required larger concentrations of NPs than in our study. This increase in NP concentration resulted in greater plasmonic coupling effects than in our study, which in turn increased the scattering cross section nonlinearly over the linear sum of the individual particle cross sections [80].

Second, NPs with a diameter larger than the expected distance between cell receptors are used. It is well-known that EGFR dimerizes (i.e., forms a molecule of two identical subunits) when bound to antibodies, as a prelude to internalization of the receptor [81]. If the diameters of the NPs are smaller than the receptor spacing (~10 nm), dimerization will lead to unwanted plasmonic coupling between NPs. However, our large-diameter NPs could each bind to two adjacent receptors so that dimerization was
associated with solely one NP, thereby preserving the scattering characteristics of an individual NP. The exact conformation is further explored in Chapter 6.

Third, the previous study used immunolabeled NPs with multiple fluorescent molecules attached to each NP [17]. Thus, when many NPs were bound to receptors close to one another, the proximity of many fluorescent molecules led to fluorescence quenching. This effect served to artificially lower the measured fluorescent intensity at high concentrations so that it no longer had a linear relationship, but instead a quadratic relationship, with receptor number. However, in this study, fluorescence and NP experiments were performed separately and avoided fluorescence quenching. For these reasons, a linear correlation between NP-scattering and receptor concentration was exhibited.

Although fluorescent tags and immunolabeled NPs were found in our study to have equivalent profiles for imaging EGFR expression, plasmonic NPs have the advantage of providing additional functionality not available with fluorescent tags. Specifically, the scattering spectra peak wavelength of NPs is dependent upon the local cellular RI, which itself reflects factors in the microscopic environment such as location within the cell and biological processes. Thus, NPs provide a potential means of revealing information regarding cellular events. It was previously shown that the alteration in the local environment posed by receptor binding produces a shift in peak wavelength [65]. In the current study, the peak wavelength of scattering by
immunolabeled NPs upon binding to EGFR varies across cell lines, even though they are expressing the same receptor. The statistically significant shift in scattering observed between NPs bound to the A431 and 270-GBM cells may be due to changes in the local nanoenvironment of the receptors. For instance, subtle changes in the consistency of the membrane could potentially affect the environment enough to shift the peak scattering wavelength. Another possible explanation for these spectra may be plasmonic coupling. If the density of EGFR on the cell membrane for a particular cell line is higher, bound NPs will be more closely spaced. This decreased spacing may result in plasmonic coupling that is observed as a slight red shift in peak scattering wavelength [17]. Further investigation is necessary to more definitively explain these differences in peak wavelength.

In summary, immunolabeled NPs can be used to quantitatively measure EGFR expression in vitro with similar performance to that of fluorescence assays, the current standard for receptor biomarkers. However, the immunolabeled NPs offer the advantages of being non-cytotoxic and immune from photobleaching, potentially enabling clinical studies and long term assays. A linear relationship was observed between receptor expression levels obtained with fluorescent markers and NP labels. NPs have the additional benefit of providing further information regarding the local RI through their scattering spectra. These findings indicate that molecular imaging using NPs may enable better characterization of cellular receptor expression than current
biomarker alternatives. Immunolabeled NPs show much potential in the advancement of molecular imaging.
5 Polarization mapping of nanoparticle plasmon coupling

In this chapter, the use of polarization as a tool to better discern the effects of plasmonic coupling from localized RI for molecular imaging and biosensing applications is investigated. Plasmonic coupling provides both a means to monitor intermolecular distances, with advantages over current techniques such as FRET, as well as an enhanced ability to sense dielectric environment. Polarization mapping isolates the transverse excitation mode that undergoes only slight plasmonic coupling, allowing for relative dielectric sensing, or absolute dielectric sensing with additional a priori interparticle distance information. First, the theoretical scattering efficiencies associated with NP pairs are modeled under parallel and orthogonal polarization orientations and increasing interparticle separation. Second, polarization mapping of substrate bound NPs using darkfield microspectroscopy is investigated as a method to isolate the individual plasmonic coupling modes associated with a pair of NPs without reorientation of the sample. The results of this study provide useful insight towards potential avenues to monitor HER-2 receptor dimerization in vitro with plasmonic NPs.
5.1 **Background**

5.1.1 **Plasmonic coupling**

As mentioned previously, the shape, composition, and local RI environment of a noble metal NP are all factors which influence the peak resonant wavelength. However, proximal NPs also play a role in its scattering and absorption properties (Figure 5.1). Plasmons between NP pairs and clusters will couple, resulting in a red shift of the peak resonant wavelength and a strong increase in the amount of scattered and absorbed light from the pair, relative to either particle individually (Figure 5.2). Plasmonic coupling is a powerful tool in many biological research applications. The enhanced scattering efficiency significantly increases biomarker contrast in molecular imaging of various sample types, including animal models of carcinogenesis [17]. Additionally, the extent of the red shift decays approximately exponentially with increasing interparticle separation [82]. This relationship allows for “molecular ruler” applications using NP dimers, including the observation of single DNA hybridization events [83, 84]. Unfortunately, coupling also has the drawback of complicating interpretation of data in dielectric sensing applications. In the presence of proximal nanospheres, changes in local RI cannot be measured accurately without *a priori* information regarding the spacing between the two particles.
Figure 5.1: SEM image of pair of 60 nm gold nanospheres on silanated coverslip. (Scale bar = 100 nm)

Figure 5.2: A) Polarization orthogonal to pair axis results in slight peak blueshift in scatter. B) Polarization parallel to pair axis results in large peak redshift in scatter.

5.1.2 Polarization effects on plasmonic coupling

Anisotropic NP targets scatter according to both their transverse and longitudinal aggregate excitation modes [19]. For instance, nanorod scattering spectra contain two distinct peaks associated with excitation of both modes, with the larger, longer wavelength peak corresponding to the longer axis. Using polarization, one can
selectively excite modes of a single orientation, resulting in only one distinct scattering peak [85]. Fortunately, plasmonic coupling is primarily dependent on excitation of the longitudinal mode parallel to the long pair axis (Figure 5.3). Polarization control therefore represents a means to isolate the two orthogonal excitation modes, which in turn can then be applied to dielectric environment sensing.

![Figure 5.3: A) Polarization orthogonal to long pair axis, causing a slight increase in repulsive forces and higher resonant frequency. B) Polarization parallel to long pair axis, weakening repulsive forces and causing a lower resonant frequency.](image)

Polarization effects on plasmonic coupling of NPs have been explored previously. Far-field polarization spectroscopy has been implemented to confirm near-field interactions between closely spaced nanosphere chains comprising waveguides [86]. Further work found that the peak resonant wavelength is polarization dependent and reflects the anisotropy of the pair [87]. As mentioned previously, Su and Schultz found that the redshift associated with elliptical nanodisc pairs decays exponentially with increased interparticle spacing. At distances past 2.5 times the particle diameter however, the shift becomes negligible [82]. Polarization significantly affects the
plasmonic coupling of NP pairs, as demonstrated by Rechberger and Aussenegg [85].

150 nm gold nanodiscs were observed at various interparticle spacings using one polarization parallel to the long axis, and a second polarization in the orthogonal orientation. The parallel polarization resulted in an observed large redshift in scattering peak resonance. The orthogonal polarization resulted in a slight blue shift. When the field is parallel to the long pair axis, the repulsive forces between the particles are weakened, which results in lower resonant frequency and a peak wavelength redshift (Figure 5.3). When the polarization of the applied field is normal to the pair axis, there are no repulsive surface charges, resulting in a higher resonant frequency, explaining the slight blue shift. These previous studies all used electron-beam lithography to fabricate the plasmonic particles, usually resulting in formation of discs, rather than spheres, with multiple crystalline domains and poorly defined perimeters [88]. NPs used as tags for molecular imaging typically have a single crystalline domain and a spherical shape. To examine plasmonic coupling for this application, it is therefore more appropriate to observe and model the behavior of similar particles produced using chemical synthesis rather than electron beam lithography.
5.2 Theoretical plasmonic coupling models

5.2.1 Theoretical polarization mapping of coupling

The ability to use polarization to map plasmonic coupling between a NP pair was evaluated using DDA simulations, as described in Section 2.3.3 [23, 24]. The first set of simulations provides a simple understanding of the changes in scattering behavior of a pair of nanospheres under orthogonal polarizations relative to one another, as well as to that of a single sphere.

Simulations begin with examination of a single 60 nm gold nanosphere that does not undergo plasmonic coupling. The sphere was modeled as a homogeneous isotropic ellipsoid (DDSCAT function ELLIPSOID [24]) and assumed to be in a dielectric environment of 1.7, similar to the environment expected for later darkfield experiments, consisting of a sphere bound to a sputtered ITO layer with an approximate RI of 2.0 and immersed in pure water medium [82, 89]. The RI was established in DDSCAT as the ratio of complex dielectric function of gold, as determined by Johnson and Christy, to the local dielectric constant [71]. DDA models included both parallel and orthogonal polarizations of incident light across a spectral range of 450 to 850 nm. As expected, both polarization models indicated identical scattering efficiency spectra for this isotropic particle. Using a peak centroid fit algorithm on the corresponding scattering efficiency, the peak resonant wavelength was determined to be 570.8 nm (Figure 5.4) [90]. The calculated efficiency peak was nearly identical to Mie theory models. Next, simulations
modeled a pair of 60 nm gold nanospheres, with 0 nm interparticle separation, as two touching homogenous isotropic spheroids (DDSCAT function SPHROID_2 [24]) which are expected to undergo a significant degree of plasmonic coupling. The same dielectric environment as the previous model is used. While the single sphere model used 1 nm inter-dipole separation in the DDA model, the case of 0 nm interparticle distance requires a finer sampling of 0.75 nm. This separation allows for a closer approximation of a true 0 nm interparticle distance while providing reasonable computation times with under $10^6$ total target dipoles (requires about 100 MB of memory [24]). In the simulations, the incident light was modeled under both parallel and orthogonal polarizations relative to the longitudinal axis of the pair. Models indicated a peak resonant wavelength of 564.2 nm for the orthogonal polarization, and 706.1 nm for the parallel polarization (Figure 5.4).

![Theoretical Comparison of Scattering from Single Nanosphere and Touching Pair](image)

**Figure 5.4:** Comparison of DDA model predicted scattering efficiency spectra from 60 nm gold nanosphere pair under $0^\circ$ and $90^\circ$ polarizations, and single nanosphere.
The second set of simulations modeled a pair of 60 nm gold nanospheres at interparticle spacing distances ranging from 0 to 90 nm (custom DDSCAT targets). Again, the local RI was established as 1.7, and the incident light was established at both parallel and orthogonal polarizations relative to the pair axis. Interparticle spacing was defined as the edge-to-edge particle distance. For nanosphere pair separations greater than 0 nm, inter-dipole separation was set to 1 nm, again to minimize computation time.

The parallel polarization simulation is summarized in Figure 5.5, indicating the scattering efficiency spectra for nanosphere pairs with increasing interparticle separation. The peak scattering efficiency wavelength exponentially decays from 706.1 nm at 0 nm interparticle separation, to 575.6 nm at 90 nm interparticle separation, close to the peak wavelength of 570.8 nm determined for the single sphere model. The exponential decay of the total redshift is clearly observed in Figure 5.5 which shows the peak scattering wavelength at increasing interparticle separations. (These results expand upon a preliminary simulation performed by Curry and Sebba [91].) The orthogonal polarization simulation is summarized in Figure 5.6, again displaying scattering efficiency spectra for nanosphere pairs of increasing interparticle separation. The peak scattering efficiency wavelength slightly shifts from 564.2 nm at 0 nm interparticle separation, to 570.5 nm at 90 nm interparticle separation, again in close agreement with the peak seen in the single sphere model. Figure 5.6 indicates the slight blue shift in peak
scattering wavelength at decreasing interparticle separations under illumination with a polarization orthogonal to the pair axis.

Figure 5.5: A) Scattering efficiency spectra of pair of 60 nm gold nanospheres under polarization parallel to pair axis and increasing interparticle separation. B) An exponential decrease in peak wavelength redshift is observed with increasing separation ($R^2 = 0.9912$).

Figure 5.6: A) Scattering efficiency spectra of pair of 60 nm gold nanospheres under polarization orthogonal to pair axis and increasing separation. B) A slight blueshift in peak wavelength is observed with decreasing separation ($R^2 = 0.9992$).

The third set of simulations models the behavior in scattering efficiencies of 60 nm gold nanosphere pairs under varying polarization orientations for three different
interparticle separations. In the case of 0 nm interparticle separation, at 0° (parallel) polarization, two distinct peaks associated with the two modes of excitation are clearly observed (Figure 5.7). As the polarization angle increases a gradual decrease in the longitudinal excitation mode peak and an increase in the transverse excitation mode peak is observed. At 90° polarization (perpendicular) only the transverse excitation mode peak remains. In the case of 6 nm interparticle separation, at 0° polarization primarily one excitation mode peak is observed. As the polarization increases, a second attenuated transverse excitation mode peak is seen to emerge (Figure 5.7). The total shift between the two excitation mode peaks has also been reduced significantly. In the final case of 30 nm interparticle separation, there are no longer two distinct peaks associated with each mode of excitation. Instead, there is simply a blue shift in peak resonance as the polarization increases towards the orthogonal orientation (Figure 5.7).

**Figure 5.7:** Scattering efficiency of pair of 60 nm gold nanospheres with 0 nm (A), 6 nm (B), and 30 nm (C) interparticle separation under polarizations 0° : 15° : 90°.
5.2.2 Increased local refractive index sensitivity

Isolation of the plasmonic coupling mode of excitation has the additional advantage of enhancement of dielectric sensing. Sensing ability is measured by the extent of the localized surface plasmon resonance (LSPR) spectral shift over the change in RI. Several methods exist for enhancing sensitivity, with usual emphasis on increasing the particle size to mass ratio [92]. Theoretical simulations by Jain et al. have demonstrated that coupling between two gold 40 nm nanospheres provides an exponential increase in medium sensitivity as interparticle distance decreases [93]. The enhancement was also experimentally verified using NP sensor arrays that demonstrated a three-fold increase in dielectric sensitivity [94].

Although the theory behind this concept has been explored previously, further simulations are carried out for the specific case of 60 nm gold nanospheres because of their wide use in the various biomarker studies described herein. Scattering efficiency spectra were modeled using DDA for three different configurations, including a pair of nanospheres under parallel and orthogonal polarizations, as well as a single particle. Refractive indices ranging from 1.55 to 1.70 (0.05 increment) were implemented in the model. A centroid peak fit algorithm determined the peak resonance wavelength [90]. Figure 5.8 indicates the peak wavelength for each RI. RI sensing capability is defined as the slope of the best linear fit for each of the three models. The single particle case had a sensing ability of 87.4 nm/RIU (refractive index units). The parallel and orthogonal
polarizations of the particle pair had sensing abilities of 230.3 nm/RIU and 75.9 nm/RI, respectively. The parallel polarization case presents a 2.6-fold increase in sensing ability over the single particle case. These results demonstrate a clear advantage in isolation of the longitudinal excitation mode of plasmonic coupling between a pair of 60 nm nanospheres.

Figure 5.8: Comparison of RI sensitivity of DDA model predicted peak scattering of both 60 nm gold nanosphere pair with 0 nm interparticle separation under parallel and orthogonal polarization, and single nanosphere. Increased slope of parallel polarization indicates increased RI sensing relative to orthogonal pair and single sphere cases.

5.3 Experimental methods

Particle Characterization

Pure 60 nm gold sphere colloid (BBInternational, Cardiff, UK) was analyzed using transmission experiments with a quartz tungsten halogen (QTH) light source (LS-1, Ocean Optics, Dunedin, FL) and a compact linear CCD spectrometer (USB2000, Ocean
Optics, Dunedin, FL). The nanospheres were determined to have a mean size of 61.4 ± 4.8 nm (N = 172) as measured using TEM (Tecnai G² Twin, FEI, Hillsboro, OR).

**Slide Preparation**

Coverslips were coated with a ~30 nm layer of indium tin oxide (ITO). Coverslips were then cleaned in piranha solution and incubated in a 10% MPTES solution. A thiol-terminated silane self-assembled monolayer forms on the surface slide allowing for binding of nanospheres.

**Spectroscopy**

A solution of 60 nm gold nanospheres was diluted to a concentration of 8.6 x 10⁹ particles/mL. 3 µL of the dilution was added to the silanated slide and allowed to bind for one minute. The unbound particles were then washed away. Water is added as an immersion medium and an additional slide is placed upon the coverslip. This configuration allows for use of an oil immersion objective and condenser to achieve darkfield illumination and detection. Scattering spectra were recorded with a previously described line imaging spectrometer setup (SpectraPro 2150i, Acton Research, Trenton, NJ)[91]. In addition, a corresponding color image was collected for spatial identification of the location of the spectral data using a color CCD (Coolsnap cf, Photometrics, Tucson, AZ).
Pairs were observed under increasing degree of detection polarization, from $0^\circ$ to $90^\circ$ (7.5° increments). Random binding of nanospheres to the silane monolayer resulted in pairs of varying interparticle distances with subsequent scattering at varying peak resonant wavelengths. For touching pairs, the polarizer was seen to eventually orient along the short axis, significantly removing the coupling-dependent peak associated with the long axis, and isolating short axis scattering. Each pair was also observed under unpolarized illumination.

**Scanning electron microscopy**

The examined slide of NPs was then observed using a scanning electron microscope (XL30 SEM-FEG, FEI, Hillsboro, OR) capable of ultra-high resolution (2-3 nm) and magnification over 300Kx to determine pair size and interparticle distances between the nanospheres. Locations were co-registered with darkfield microscopy images using mesh reference grids (G400-Ni, EMS, Hatfield, PA).

**Data Analysis**

Data were acquired past $0^\circ$ and $90^\circ$ orientations to ensure complete characterization of the two excitation modes. Both theoretical and experimental peaks were analyzed using a polynomial fit peak centroid determination algorithm in Matlab that provides increased shift precision [90].
5.4 Polarization mapping of coupling

5.4.1 Case 1: Proximal nanosphere pair

In the case of proximal nanosphere pairs, polarization mapping discerned two distinct excitation mode peaks, with large spectral intervals between the two. For example, in Figure 5.9 a nanosphere pair with 3 nm interparticle separation is observed using SEM. The same pair is also observed using darkfield illumination where a large visible shift in peak scattering between the two polarizations is seen. The shift is then quantified by microspectroscopy. Under the parallel and orthogonal polarizations, centroid peaks of 703.7 nm and 559.8 nm are observed, respectively, representing a shift of 143.9 nm. These experimental findings are in close agreement to DDA models of a similar proximal nanosphere pair, with 4 nm interparticle separation (Figure 5.10). Under the parallel and orthogonal polarizations, centroid peaks of 694.1 nm and 565.4 nm are seen, respectively, representing a shift of 128.7 nm.

Figure 5.9: A) SEM image of observed 60 nm gold nanosphere pair with 3 nm interparticle separation (Scale bar = 100 nm). Same NP pair observed under darkfield illumination at polarization orthogonal (B) and parallel (C) to pair axis (Scale bar = 1.5 μm).
5.4.2 Case 2: Nanosphere pair with large interparticle separation

In the case of nanosphere pairs with large interparticle separation, polarization mapping no longer discerned two distinct excitation mode peaks, but rather a single peak that slightly blue shifted with increasing degree of polarization. For example, in Figure 5.11 a nanosphere pair with 41 nm interparticle separation is seen using SEM. The same pair is also observed using darkfield illumination. Darkfield images exhibit only a small visible shift in peak scattering between the two polarizations. The shift is then quantified by microspectroscopy. Under the parallel and orthogonal polarizations, centroid peaks of 577.8 nm and 562.8 nm are observed, respectively, representing a shift of 15.0 nm. These experimental findings are in close agreement to DDA models of a similar distant nanosphere pair, with 41 nm interparticle separation (Figure 5.12). Under
the parallel and orthogonal polarizations, centroid peaks of 583.4 nm and 569.3 nm are seen, respectively, representing a shift of 14.1 nm.

Figure 5.11: A) SEM image of observed 60 nm gold nanosphere pair with 41 nm interparticle separation (Scale bar = 100 nm). Same NP pair observed under darkfield illumination at polarization orthogonal (B) and parallel (C) to pair axis (Scale bar = 1.5 µm).

Figure 5.12: A) DDA model of scattering efficiency of 60 nm nanosphere pair with 45 nm interparticle separation under 0° and 90° polarizations. B) Experimental scattering spectra of pair shown in Figure 5.11 with 41 nm interparticle separation under 0° and 90° polarization.

Observation of nanosphere pairs over a larger distribution of interparticle separations provides further evidence of the ability to separate the excitation modes using polarization. Experiments additionally demonstrate the relationship between peak scattering wavelength of both the parallel and orthogonal polarizations and interparticle
separation of all nanosphere pairs (N= 42) (Figure 5.13). Under parallel polarization, the expected exponential decay in peak redshift ($R^2 = 0.9203$) predicated by DDA simulations is seen. An observable trend with decreasing interparticle separation is more difficult to discern in the orthogonal polarization peaks ($R^2 = 0.0023$). For this case, DDA simulations indicated a slight blue shift in peak wavelength with decreasing interparticle separation. The largest contributor to this behavior appears to be the size distribution of the particles themselves.

![Figure 5.13: Experimental peak scattering of approximately 60 nm gold nanospheres at 0° (red) and 90° (blue) polarizations under increasing interparticle separation with $R^2 = 0.9203$ and 0.0023, respectively.](image)

To account for this size distribution, the total spectral shift between the two excitation mode peaks is normalized by the expected single sphere scattering peak using Mie theory ($\lambda_0 + \Delta\lambda / \lambda_0$). Figure 5.14 shows an exponential decay relationship between total spectral shift and increasing interparticle separation ($R^2 = 0.9234$). Again, peaks of
NP pairs at larger interparticle spacing distances approach the single sphere case of scattering, although closer spheres are exponentially red-shifted. The theoretical decay relationship as determined through DDA simulation appears to be in close agreement with the experimental findings.

![Peak Shift at Increasing Interparticle Separation](image)

**Figure 5.14:** Experimental peak shift between 0° and 90° polarizations of approximately 60 nm gold nanospheres under increasing interparticle separation, normalized by Mie theory predicted single sphere peak scattering wavelength to account for size distribution. Fit is compared to DDA model of 60 nm gold nanosphere relationship.

### 5.5 Discussion: Isolation of excitation modes

Both experimental results and theoretical simulations demonstrate the ability to separate the longitudinal and transverse excitation modes using polarization mapping. Theoretical simulations clearly indicate a distinct difference between the two excitation modes, particularly with decreased interparticle separation. Experimental data shows separation of the peaks using polarization mapping in close agreement with these theoretical results.
The best fit of experimental data comparing peak scattering of the longitudinal excitation mode and interparticle separation demonstrated an $R^2 = 0.9234$. While this goodness of fit provides an important indication of the general relationship, it stops short of providing an experimental calibration at a high enough level to permit nanometer scale ruler functions. This observation is attributed to both the size distribution of the particles, as mentioned previously, and a slight decrease in resolution of SEM size measurements due to overcharging of the glass substrate, despite the conductive ITO coating. Also, the implemented polarizer increment of $7.5^\circ$ represents only an approximation of the true transverse modes of excitation.

Both theoretical and experimental results on single crystalline domain 60 nm gold nanospheres are in close agreement with previous studies that explored plasmonic coupling between electron beam lithography-formed ellipsoid disks. Both groups experimentally found similar relationships between interparticle separation and peak scattering efficiency wavelength for both the longitudinal and transverse excitation mode cases [85, 95]. The results presented here demonstrate that single crystalline domain spheres exhibit the same behavior of ellipsoidal disks formed using electron beam lithography.

While the ability to discern the two modes using polarization have been shown, theoretical simulations clearly indicate that the isolated transverse excitation mode will still exhibit plasmonic coupling effects, albeit small. This effect, which results in the
slight blue shift of peak scattering efficiency wavelength, can be attributed to the charge
distribution on the proximal sphere affecting the resonance of the pair together [85]. The
maximum theoretical blue shift is only 6.6 nm in the 0 nm interparticle separation case,
but becomes negligible at larger distances (0.3 nm at 90 nm interparticle separation).

Because the transverse excitation mode remains largely dependent on
interparticle distance, it cannot be used to reveal absolute RI without additional
information regarding the spacing between NPs. Therefore, there are two possible
approaches for dielectric sensing using particle pairs. The first method allows for
relative sensing based on the case where the absolute interparticle separation is
unknown. If it is known that the interparticle distance will vary within a small limited
range, the blue shift will actually be limited to only a couple nanometers. This approach
will indicate local RI, but with an increased error in measurement. If the absolute
distance is unknown, but constant throughout the population of pairs, again relative
sensing will be appropriate. A second approach involves gaining a priori information
regarding the interparticle separation. Once this distance is known, absolute sensing
with high sensitivity becomes possible.

This investigation has direct implications in molecular imaging and biosensing
applications. Based on these findings, the monitoring of receptor dimerization and the
corresponding dielectric environment using immunolabeled nanospheres may require a
priori interparticle separation information. This application and the potential advantages of polarization mapping in vitro are further explored in Section 6.4.
6 Molecular imaging of multiple receptor targets

In Chapter 6, for the first time simultaneous multiplex detection of three different types of plasmonic NPs is demonstrated, with each targeted to a different receptor commonly overexpressed in metastatic cancers, including anti-EGFR gold nanorods, anti-IGF-1R silver nanospheres, and HER-2 Ab gold nanospheres. This study also represents the first time in which the specific combination of anti-IGF-1R has been conjugated to silver nanospheres, and HER-2 Ab to gold nanospheres. Each label was chosen to scatter strongly in a separate spectral window. The ability to monitor all of these receptors simultaneously will provide a better understanding of the mechanisms in which these different receptor signaling pathways interact or potentially open up new avenues of photothermal therapy. Second, molecular specificity of all three tags is established. Third, using a combination of Mie theory and DDA, the local RI of the cellular membrane binding these labels is determined. In the case of HER-2 receptors, the conformation of proximal NPs bound to receptors undergoing dimerization is also investigated. Finally, simultaneous dual and triple tag experiments demonstrate the ability to profile the immunophenotype of various cell lines.
6.1 Methods

6.1.1 Nanoparticle Conjugation

6.1.1.1 Anti-EGFR nanorod conjugation

Gold nanorods were synthesized using established seed-mediated methods [96]. To an aqueous mixture of 7.5 mL of 0.1 M CTAB (Sigma-Aldrich, St. Louis, MO) and 0.250 mL of 0.01 M hydrogen tetrachloroaurate trihydrate (HAuCl₄, Sigma-Aldrich, St. Louis, MO), 0.6 mL of ice cold 0.01 M sodium borohydride (Sigma-Aldrich, St. Louis, MO) was added under vigorous stirring to produce the gold seed, which was then gently heated and stirred for a few minutes. To a separate bottle containing 95 mL of 0.1 M CTAB in water at 29°C (Precision microprocessor-controlled 280 series water bath), 4 mL of 0.01 M HAuCl₄, 0.6 mL of 0.01 M silver nitrate (Sigma-Aldrich, St. Louis, MO), and 0.64 mL of 0.1 M ascorbic acid (Sigma-Aldrich, St. Louis, MO) were added, and the mixture was swirled after the addition of each reactant. After the addition of 50 µL of gold seed, the bottle was capped, inverted five times, and incubated at 29°C overnight. The dimensions of the gold nanorods were measured at 67.1 ± 8.9 nm in length and 32.0 ± 6.1 nm in diameter (n=114) using TEM (Tecnai G² Twin, FEI, Hillsboro, OR) (Figure 6.1). Assuming a perfectly cylindrical nanorod shape, as well as the complete conversion of HAuCl₄ to Au, the concentration of the parent gold nanorod suspension was estimated to be 1.3⋅10⁻¹⁰ M.

Gold nanorods were conjugated according to published procedures with anti-EGFR (E2156, clone 225, Sigma-Aldrich, St. Louis, MO, 1.56 mg/mL stock solution)
or with mouse IgG1 κ isotype control (16-4714-85, eBioscience, 1.54 mg/mL stock solution) as a negative control, as follows: 1 mL of the gold nanorod suspension was centrifuged twice (10,000 RPM for 5 min.) and resuspended in 1 mL of 1 mM NaCl [97]. Polystyrene sulfonate (200 µL, 10 mg/mL in 1 mM NaCl, MW 18,000, Polysciences, Inc.) was added to the nanorods, and the suspension was placed on a shaker for 20 min. The nanorods were centrifuged at 10,000 RPM for 5 min. and resuspended in 1 mL of 20 mM HEPES pH 7.4. Four µL of 1.56 mg/mL mouse monoclonal anti-EGFR were added, and the suspension was placed on an oscillator for 30 min. Antibody concentrations were determined using an NanoDrop Spectrophotometer (ND1000, Nanodrop, Wilmington, DE). Optimal antibody concentration was determined by testing for suspension stability (i.e., least aggregation) under various amounts. The nanorods were centrifuged at 10,000 RPM for 5 min. and then finally resuspended in 0.5 mL of PBS containing 5 mg/mL BSA. This protocol is an adaptation of methods developed by the El-Sayed group [97].

Figure 6.1: (Left) TEM images of uncoated gold nanorods indicating average size of 32.0 ± 6.1 nm x 67.1 ± 8.9 nm (N=114, Scale bar = 100 nm). (Right) Anti-EGFR nanorods indicating antibody thickness of 5.5 ± 1.3 nm (N=30, Scale bar = 50 nm).
6.1.1.2 Anti-IGF-1R nanosphere conjugation

Anti-IGF-1R nanosphere conjugates consist of 0.5 mL of 100 nm diameter silver colloid (15709-20SC, Ted Pella, Inc., Redding, CA) solution (Figure 6.3) diluted with 485 µL of 20mM HEPES buffer and 16.1 µL of 1.40 mg/mL anti-IGF-1R (MCA2344, ABD Serotec, Raleigh, NC) solution diluted with 62.5 µL of 20mM HEPES buffer. 100 nM K$_2$CO$_3$ was used to adjust the pH of each solution to 7.0 ± 0.2. Solutions were then mixed on an oscillator for 20 minutes at 190 cycles/min. Following mixing, the solution was tested for antibody-nanoparticle conjugation by removing 100 µL and subsequent addition of 5 µL of 10% NaCl. Incomplete antibody coverage would result in a bluish color change due to particle aggregation. To prevent non-specific binding of proteins to remaining NP surface, 100 µL of 1% PEG (P2263, Sigma-Aldrich, St. Louis, MO) was added to the suspension and then allowed to interact. 15 minutes of centrifuging at 6000
RPM was used to remove excess PEG. Supernatant was removed, and the NP pellet was resuspended with 0.5 mL of PBS. These steps were then repeated to ensure complete removal of PEG, with minimal removal of NPs (Figure 6.3). This protocol is an adaptation of methods developed by the Richards-Kortum and Sokolov groups [29, 98].

**Figure 6.3:** (Left) TEM image of uncoated silver nanospheres indicating average size of 99.3 ± 12.0 nm (N=101, Scale bars = 100 nm). (Right) Anti-IGF-1R silver nanospheres indicating antibody layer thickness of 4.5 ± 1.4 nm (N=80).

**Figure 6.4:** Extinction spectrum of uncoated silver nanospheres in suspension with a primary peak of 483.5 ± 0.5 nm.
6.1.1.3 HER-2 Ab nanosphere conjugation

HER-2 Ab nanosphere conjugates consist of 0.5 mL of 60 nm diameter Au colloid (15709-20, Ted Pella, Inc., Redding, CA) solution (Figure 6.5) diluted with 485 µL of 20mM HEPES buffer and 14.4 µL of 1.04 mg/mL HER-2 Ab (MS-301-PABX, Labvision, Fremont, CA) solution diluted with 62.5 µL of 20mM HEPES buffer. 100 nM K$_2$CO$_3$ was used to adjust the pH of each solution to 7.0 ± 0.2. Solutions were then mixed on an oscillator for 20 minutes at 190 cycles/min. Following mixing, the solution was tested for antibody-nanoparticle conjugation by removing 100 µL and subsequent addition of 5 µL of 10% NaCl. Incomplete antibody coverage would result in a bluish color change due to particle aggregation. To prevent non-specific binding of proteins to remaining NP surface, 100 µL of 1% PEG (P2263, Sigma-Aldrich, St. Louis, MO) was added to the suspension and then allowed to interact. 15 minutes of centrifuging at 6000 RPM was used to remove excess PEG. Supernatant was removed, and the NP pellet was resuspended with 0.5 mL of PBS. These steps were then repeated to ensure complete removal of PEG, with minimal removal of NPs (Figure 6.5). This protocol is an adaptation of methods developed by the Drezek, Richards-Kortum, and Sokolov groups [29, 31, 98].
6.1.2 Preparation of cell cultures

EGFR (+) / IGF-1R(-) / HER-2(-) MDA-MB-468 human breast adenocarcinoma were incubated at 37°C and 5% CO₂ using MEM Alpha Medium, with 10% fetal bovine serum 1% penicillin streptomycin [60, 99, 100].
HER-2(+) SK-BR-3 human breast adenocarcinoma were incubated at 37° C and 5% CO₂ using McCoy’s 5A Medium Modified, with 10% fetal bovine serum 1% penicillin streptomycin [100, 101].

EGFR (+) / IGF-1R(low) / HER-2(low) expressing A549 human alveolar adenocarcinoma cells were incubated at 37° C and 5% CO₂ using F-12 Nutrient Mixture (Ham), 10% fetal bovine serum and 1% penicillin streptomycin. A second population of FACS selected high IGF-1R expressing A549 cells. A third population of A549 cells were additionally transfected with pcDNA 3.1 HER-2 and FACS selected for high HER-2 expression. (See Section 6.1.3 for further information.)

EGFR (-) / IGF-1R(-) / HER-2(+) MDA-MB-453 human breast carcinoma cells were incubated at 37° C using Leibovitz L-15 Medium, with 10% fetal bovine serum and 1% penicillin streptomycin [60, 100, 101].

IGF-1R(low) / HER-2(-) MCF-7 human breast adenocarcinoma cells were incubated at 37° C and 5% CO₂ using MEM Alpha, 10% fetal bovine serum and 1% penicillin streptomycin [60, 102, 103].

IGF-1R(-) / HER-2(+) BT-474 human breast carcinoma cells were incubated at 37° C and 5% CO₂ using DMEM, 10% fetal bovine serum and 1% penicillin streptomycin [60, 102, 103].
EGFR (-) / IGF-1R(low) / HER-2(-) COLO-320DM human colon adenocarcinoma were incubated at 37° C and 5% CO\textsubscript{2} using RPMI Medium 1640, 10% fetal bovine serum and 1% penicillin streptomycin [100].

All cell lines were obtained from the ATCC through the Duke Cell Culture Facility.

**Table 6.1: Relative expression levels of implemented cell lines**

<table>
<thead>
<tr>
<th>Expression: [ EGFR</th>
<th>IGF-1R</th>
<th>HER-2 ]</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-468</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MCF-7</td>
<td>NA</td>
<td>low</td>
</tr>
<tr>
<td>BT-474</td>
<td>NA</td>
<td>-</td>
</tr>
<tr>
<td>COLO-320DM</td>
<td>-</td>
<td>low</td>
</tr>
<tr>
<td>A549</td>
<td>+</td>
<td>low</td>
</tr>
</tbody>
</table>

### 6.1.3 Gene transfection

Direct immunofluorescence experiments using anti-HER-2/neu FITC (340553, BD Biosciences, San Jose, CA) indicated negligible levels of HER-2 expression in EGFR / IGF-1R expressing A549 breast human alveolar adenocarcinoma cells. A gene transfection was therefore performed on these cells using a pcDNA3.1/HER-2 vector (courtesy of Dr. Gudrun Huper). Four T75 flasks were plated at 10\textsuperscript{6} cells and left to grow overnight. The flasks were designated for pcDNA3.1/HER-2, and three controls including pcDNA3.1, green fluorescent protein (GFP), and an un-transfected population. 10 µg of DNA was used for pcDNA3.1, pcDNA3.1/HER-2, and GFP. 500 µL of Opti-MEM (11058-021, Invitrogen, Carlsbad, CA) was added to 30 µL of Fugene 6 transfection
reagent (11815091001, Roche Applied Science, Indianapolis, IN). The solution was left to sit for 5 minutes at room temperature. The appropriate amount of DNA was then added and again allowed to sit at room temperature for 15 minutes. The Fugene/DNA complex was then added to the appropriate flask of cells. Two days later the flasks of cells were split and plated to two large culture dishes. The media used contained 50 µg/mL of geneticin to select for the geneticin resistance gene. After about two weeks of growth, the HER-2 transfected plates were pooled.

 Successful transfection of the pooled cells was confirmed through Western Blot analysis using established techniques (Figure 6.7), as previously described in Section 4.1. In this instance, the primary antibody implemented was rabbit monoclonal antibody (1:1000, clone 29D8, 2165, Cell Signaling Technology, Danvers, MA) and the secondary antibody was donkey anti-rabbit IgG-HRP (1:25,000, sc-2077, Santa Cruz Biotechnology, Santa Cruz, CA). The internal control GAPDH was detected using goat polyclonal anti-GAPDH antibody (1:2000, sc-20357, Santa Cruz Biotechnology, Santa Cruz, CA). Results confirmed an expected molecular weight of 185 kDa and indicated low levels of expression in both the transfected and original cells, which was unexpected given original direct immunofluorescence results. Nonetheless, previous studies do confirm these findings in A549 cells [60]. To increase HER-2 expression, FACS was used to sort the top 5% of HER-2 expressing pooled transfected cells, as described below in Section 6.1.4.
Figure 6.7: Western Blot probing for HER-2 expression in A) A549 HER-2 transfected cells (pooled), B) A549 pcDNA3.1 control cells (pooled), C) A549 untransfected control cells, and D) BT-474 HER-2 positive control cells. Bottom image shows GAPDH loading control.

6.1.4 Fluorescence-activated cell sorting

FACS was conducted to increase receptor expression in two cell populations, including A549 cells which inherently express mid-levels of IGF-1R, and HER-2 gene transfected A549 cells with low levels of HER-2. 5×10^6 cells were prepared for sorting, in addition to 10^6 cells each for IgG and unlabeled control. Cells designated for sorting were incubated with either 20 μL of anti-HER-2/neu FITC (340553, BD Biosciences, San Jose, CA) or 10 μL mouse anti-human CD221 (anti-IGF-1R) Alexa Fluor 488 (MCA2344A488T, AbD Serotec, Raleigh, NC) per 10^6 cells. Cells designated for IgG controls were incubated with identical concentrations of IgG conjugates (mouse IgG\textsubscript{i} FITC (555748, BD Biosciences, San Jose, CA) or mouse IgG\textsubscript{i} negative control Alexa Fluor
488 (MCA928A488, AbD Serotec, Raleigh, NC) at identical cell suspension concentrations. All steps were performed in minimal light to prevent photo-bleaching. Cell populations were placed on an oscillator for 22 minutes at 100 rotations/s. Tubes were then spun down for 2 minutes at 1200 RPM. Excess supernatant was removed and cells were washed with 2%FBS/PBS solution. Wash steps were then repeated a second time, and cells were resuspended in the 2%FBS/PBS solution at a concentration of $10^7$ cells/mL to ensure optimal sorting times. All cell populations were then covered in foil and placed in the refrigerator. Sorting was conducted within a 3 hour window. Following sorting, as appropriate penicillin and geneticin media supplements were doubled to prevent contamination. In the case of HER-2 gene transfected A549 cells, the top 5.18% of HER-2 expressers were sorted (Figure 6.8A). In the case of IGF-1R expressing A549 cells, the top 13.17% of IGF-1R expressers were sorted for IGF-1R molecular specificity experiments (Figure 6.8B).

Figure 6.8: A) FACS of A549 cells for top 5.18% of HER-2 expressers; B) FACS of A549 cells for top 13.17% of IGF-1R expressers.

90
Although well-characterized cell lines were implemented in labeling experiments, further confirmation of receptor expression levels was performed using established methods. One technique, enzyme-linked immunosorbent assay (ELISA) detects and quantifies the presence of a particular cellular protein with high sensitivity. Membrane preparations were performed to analyze proteins localized to the cell membrane. Subsequent BCA protein assays were conducted to ensure equal protein loading. All implemented cell lines were screened in triplicate for EGFR (Table 6.1), IGF-1R (Table 6.2), and HER-2 (Table 6.3) using commercially available kits including the EGFR (Full-length) Human ELISA Kit (KHR9061, Invitrogen, Carlsbad, CA), STAR IGF-1R ELISA Kit (17-481, Millipore, Billerica, MA), and Human HER-2 immunoassay Kit (Total) (KHO0701, Invitrogen, Carlsbad, CA), respectively. In the case of EGFR and HER-2, a 1:100 dilution of 1 µg of membrane protein was loaded to ELISA wells. For IGF-1R, a 1:20 dilution of 1 µg of membrane protein was loaded due to lower relative expression levels. All cell lines were characterized within 5 passages from the time of original labeling experiments.

Determination of cut-off between negatives and positives is determined by “mean + 3·(standard deviation)” of known negative expressing cell line. The cut-off for EGFR expression is 13.44 ng/mL, classifying MDA-MB-468 [60, 99, 100], A549/HER-2, and A549 [104] as positives and COLO-320DM [100] and MDA-MB-453 [60, 100] as negatives, which is in agreement with previous studies. The cut-off for IGF-1R trials is
1.65 ng/mL, classifying all cell lines (MCF-7 [60, 102], BT-474 [60, 102], COLO-320DM, A549 [60, 105], A549 / HER-2, and MDA-MB-453 [60]) as low expressing positives, with the exception of the negative MDA-MB-468 [60, 106], which is in agreement with previous studies. The cut-off for HER-2 trials is 50.94 ng/mL, classifying BT-474 [60, 102, 103], SK-BR-3 [100, 101], and MDA-MB-453 [60, 100, 101] as positives and A549/HER-2, COLO-320DM [100], and MDA-MB-468 [101, 103, 107] as negatives, which is in agreement with previous studies. The classification of the A549 / HER-2 cell line as a negative rather than a low expresser is contradicted by flow cytometry findings exhibited during FACS.

Table 6.2: Expression of EGFR protein as measured by ELISA

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>EGFR Protein (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-468</td>
<td>55.96 ± 3.68</td>
</tr>
<tr>
<td>A549 / HER-2</td>
<td>22.97 ± 3.90</td>
</tr>
<tr>
<td>A549</td>
<td>16.09 ± 1.94</td>
</tr>
<tr>
<td>COLO-320DM</td>
<td>12.56 ± 0.62</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>10.98 ± 0.82</td>
</tr>
</tbody>
</table>

Table 6.3: Expression of IGF-1R protein as measured by ELISA

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IGF-1R Protein (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>6.55 ± 1.12</td>
</tr>
<tr>
<td>BT-474</td>
<td>6.32 ± 0.40</td>
</tr>
<tr>
<td>COLO-320DM</td>
<td>3.70 ± 0.55</td>
</tr>
<tr>
<td>A549 / IGF-1R</td>
<td>3.53 ± 0.68</td>
</tr>
<tr>
<td>A549 / HER-2</td>
<td>2.75 ± 3.10</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>2.40 ± 0.26</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>0.90 ± 0.25</td>
</tr>
</tbody>
</table>
Table 6.4: Expression of HER-2 protein as measured by ELISA

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HER-2 Protein (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT-474</td>
<td>2027.85 ± 75.94</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>439.30 ± 14.98</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>51.44 ± 10.81</td>
</tr>
<tr>
<td>MCF-7</td>
<td>45.88 ± 9.97</td>
</tr>
<tr>
<td>A549 / HER-2</td>
<td>38.45 ± 7.40</td>
</tr>
<tr>
<td>COLO-320DM</td>
<td>34.36 ± 4.74</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>21.95 ± 9.66</td>
</tr>
</tbody>
</table>

6.1.5 Spectral Measurements

Cell treatment with nanoparticles

After overnight incubation of chambers plated with 80K cells, media was removed from cells designated for experiments. A solution consisting of 0.5 mL PBS / 5% FBS and 0.5 mL NP conjugate suspension was then added. Next, cells were incubated for 20 minutes, allowing for adequate interaction between conjugates and cells. After removal of the solution, cells were washed twice with media. In the case of dual and triple tag labeling, this procedure was then repeated with the addition NP conjugates. Experiments were then immediately conducted using the hyperspectral microscopy system.
**Nanoparticle spectral measurement**

The hyperspectral microscopy system described in Chapter 3 was used to conduct experiments on cells labeled with NP antibody conjugates. Hyperspectral data cubes were acquired from 450-700 nm, with individual images integrating for 30 ms. Each cell was imaged in a 170 x 170 pixel region of interest to minimize acquisition time. Two data cubes were acquired for each cell, with 5 and 1 nm increments, taking a total acquisition time of ~2.4 and ~12.0 s, respectively. Source-correction is performed by normalizing the signal by source intensity, as measured using a diffuse reflectance standard (WS-1, Ocean Optics, Dunedin, FL). Transmission experiments were conducted on both uncoated and immunolabeled conjugates using a spectrophotometer (Varian 300, Cary, Santa Clara, CA) with deuterium UV and mercury visible lamps.

### 6.2 Individual plasmonic label dielectric sensing

In this section, experiments are presented that investigate cellular dielectric sensing using two novel plasmonic NPs. The RI of the cellular environment provides a potential tool for examining a variety of biological processes. For instance, monitoring changes in intracellular solute and protein concentration can indicate pathology or cellular function. Proximal to a plasmonic NP, even slight changes in these concentrations will produce a detectable dielectric-dependent shift in the peak wavelength of the surface plasmon resonance. Studies have already demonstrated this
correlation, with observation of hypotonic stress [108], oxygen saturation levels [109, 110], and mitochondrial membrane potential [111]. In addition, adjacent cellular structures can also greatly affect the local dielectric environment, indicating NP location, intercellular organization, and changes in intracellular architecture [112]. Demonstration of this application includes the observation of the formation of endocytic organelles and various reagent effects on cell morphology [113, 114].

Several methods have been explored for determination of cellular RI. Early techniques were based on a combination of interference microscopy and immersion refractometry, a method that relies on serial immersion of graded RI solutions to obtain dielectric matching [115]. The technique is limited by extensive experimental steps and eventual cell destruction [116]. Recent work has focused on using a variety of phase microscopy implementations to indicate cellular RI. One technique uses confocal microscopy to establish cell thickness and then quantitative phase microscopy for determination of phase difference [116]. Another approach determines the optical path length by confining cells in microfluidic chambers with controlled height [110]. These approaches suffer from drawbacks that include spatial averaging across regions of the cell and the inability to probe local intracellular RI [16].

Surface plasmon resonance-based (SPR) biosensors show potential in probing intracellular dielectric environment. For instance, one study monitored changes in RI associated with intracellular reactions in response to cell ligand stimulation [117].
However, these biosensors suffer from the inability to detect intracellular RI distributions and only provide large sensing distances on the order of hundreds of nanometers due to the nature of evanescent field layer sensing. An improved configuration based on SPR imaging is capable of detecting these intracellular distributions, but again only measures over large sensing distances [118]. Nonetheless, these systems do have applications for large throughput analyses of cell populations.

A recent alternative approach for intracellular RI sensing uses the localized surface plasmon resonance of NPs on a more local scale (tens of nanometers) [16]. In the first study to demonstrate this technique, 60 nm gold nanospheres were immunolabeled with anti-EGFR antibody. EGFR over-expressing A431 cells were exposed to the NP conjugates and then monitored with darkfield molecular imaging and microspectroscopy. The study first demonstrated molecular specificity of the label, which was based on a protocol developed by Sokolov et al [29]. Extinction measurements of pure and anti-EGFR coated nanosphere colloid allowed for determination of local intracellular RI from the peak scattering wavelength of exposed cells. Peak scattering at 564.8 ± 5.0 nm indicated an average local RI of 1.53 ± 0.02 in the volume surrounding the particle [98].

Previous studies by our group have established the benefits of plasmonic NPs in molecular imaging, including measurement of receptor expression and sensing of local RI environments. While these works focused on anti-EGFR labeled 60 nm gold spheres,
there is nothing limiting this technique from application to either alternative antibodies or NPs. Various spectrally distinct NPs can easily be fabricated by altering their shape, size, or composition. Several types of labels can therefore be used simultaneously as long as they are chosen to fall within different spectral windows. For instance, silver nanospheres, gold nanospheres, and gold nanorods all have relatively distinct scattering profiles over the visible spectrum (Figure 6.9). Functionalizing these distinct NPs with various biologically relevant antibodies will allow monitoring of multiple cellular receptors simultaneously. In this study, anti-EGFR gold nanorods and anti-IGF-1R silver nanospheres are implemented to sense cellular RI in cell lines known to over-express their respective receptor.

Figure 6.9: Extinction spectra of uncoated 67 x 32 nm gold nanorod, 60 nm gold nanosphere, and 100 nm silver nanosphere colloid suspensions.
6.2.1 Anti-EGFR gold nanorods

6.2.1.1 Molecular Specificity

Experiments were performed to demonstrate the molecular specificity of the anti-EGFR gold nanorod conjugation protocol. MDA-MB-468 human breast adenocarcinoma cells over-expressing EGFR were exposed to the anti-EGFR conjugates [60, 99, 100]. Hyperspectral data cubes were acquired for individual cells using the hyperspectral microscopy system previously described in Chapter 3. Scattering spectra averaged over the entire cell were fit to Gaussian distributions, indicating an average peak scattering wavelength of 664.0 $\pm$ 9.3 nm for this label (N=139) (Figure 6.10).

![Figure 6.10: Representative image (left) and spectrum (middle) of MDA-MB-468 cell bound with anti-EGFR gold nanorods. (Scale bar = 10 µm)
Figure 6.11: Control experiments consisting of (left) EGFR expressing MDA-MB-468 cells exposed to IgG Ab nanorods, (middle) EGFR expressing MDA-MB-468 cells not exposed to anti-EGFR nanorods, and (right) EGFR non-expressing MDA-MB-453 cells exposed to anti-EGFR nanorods. Both images and spectra normalized identically to scale shown in Figure 6.10. (Scale bars = 10 µm)

Controls consisted of exposure of EGFR non-expressing MDA-MB-453 human breast carcinoma cells to anti-EGFR nanorod conjugates [60, 100], EGFR expressing MDA-MB-468 cells exposed to IgG1 Ab labeled gold nanorods, and unexposed MDA-MB-468 cells (Figure 6.11). Control experiments showed negligible increases in scattering, demonstrating statistically significant differences in scattering intensity (p < 0.001) and molecular specificity (Figure 6.12).
Figure 6.12: Mean scattering intensity of 0.111 ± 0.065 (N= 139) for EGFR expressing MDA-MB-468 cells exposed to anti-EGFR nanorods (+/+), 0.017 ± 0.005 (N=50 ) for MDA-MB-468 cells exposed to IgG Ab nanorods (+ / IgG), 0.017 ± 0.004 (N=50 ) for MDA-MB-468 cells unexposed to conjugates (+ / 0), and 0.020 ± 0.005 (N= 50) for EGFR non-expressing MDA-MB-453 cells exposed to anti-EGFR nanorods (- / +).

6.2.1.2 Refractive index sensing

Determination of the local membrane RI using anti-EGFR gold nanorods requires three separate steps. First, the size distribution of the nanorods are measured to be 67.1 ± 8.9  nm x 32.0 ± 6.1  nm with hemispherical ellipsoidal caps with radii of 10.3 ± 3.1 nm using TEM (N=114). Second, the coated antibody layer thickness of anti-EGFR coated gold nanorods were measured using TEM and determined to be 5.5 ± 1.3 nm (N=30). Using the antibody size distribution and thickness, in addition to the extinction peak of 641.5 ± 0.5  nm observed for the coated nanorods, DDA theory was used to model the rods as two concentric rods with ellipsoidal caps of distinct dielectric constant [23, 24, 71]. The model indicated an antibody layer RI of 1.44 ± 0.02. Finally, using the mean
peak scattering wavelength of 664.0 ± 9.3 nm (N=139, SEM = 0.8) (Figure 6.13), in addition to the derived parameters above, DDA models can be used to determine the local RI at the cell membrane in the volume around the label to be 1.49 ± 0.03. (The sensing distance of the nanorods is approximately 40 nm from the surface as estimated by Marinakos, et al. on similarly sized NPs [67].)

Table 6.5: Refractive index calculation parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoated Particle Size (TEM)</td>
<td>67.1 ± 8.9 x 32.0 ± 6.1, 10.3 ± 3.1 nm</td>
</tr>
<tr>
<td>Antibody Layer Thickness (TEM)</td>
<td>5.5 ± 1.3 nm</td>
</tr>
<tr>
<td>Coated Colloid Extinction Peak</td>
<td>641.5 ± 0.03 nm</td>
</tr>
<tr>
<td>Antibody Layer RI</td>
<td>1.44 ± 0.02</td>
</tr>
<tr>
<td>Cell Bound Scatter Peak</td>
<td>664.0 ± 0.8 nm</td>
</tr>
<tr>
<td>Cellular Environment RI</td>
<td>1.49 ± 0.03</td>
</tr>
</tbody>
</table>

Figure 6.13: Histogram of peak scattering peak wavelengths of MDA-MB-468 cells (N = 150) bound with anti-EGFR gold nanorods (67.1 ± 6.1 x 32.0 ± 8.9 nm) Distribution fit has peak wavelength of 664.0 ± 9.3 nm.
6.2.1.3 Uncertainty determination

The uncertainty of the local cellular RI measurement is determined using the methods outlined by Curry et al. [16, 119]. Calculation of total uncertainty requires the measurement and orthogonal summation of each of the uncertainties associated with the individual parameters. The determination of peak scattering wavelength of cells bound to anti-EGFR gold nanorods has an uncertainty of 0.8 nm, as determined by the standard error of mean of cell scatter peak measurements. The mean nanorod lengths have an uncertainty of 0.70 nm as determined by the standard error of mean of the TEM measurements (STD = 7.5, N = 114). The antibody layer thickness has an uncertainty of 1.46, representing the summation of its respective uncertainties. Finally, the antibody layer RI has an uncertainty of 0.02 RIU, also representing the summation of its respective uncertainties. Each of these uncertainties is then multiplied by their respective differential relationship with the cellular RI, as seen in equation 6.1, where Δr is the particle size uncertainty, Δt is the antibody layer thickness uncertainty, Δn_{coat} is the antibody layer RI uncertainty, Δλ_{sca} is the cell scatter uncertainty, and n_{cell} is the cellular RI uncertainty [16]. From this summation, the total uncertainty is calculated to be 0.03 nm.

\[
\Delta n_{cell}^2 = \left(\frac{\partial n_{cell}}{\partial r} \cdot \Delta r\right)^2 + \left(\frac{\partial n_{cell}}{\partial t} \cdot \Delta t\right)^2 + \left(\frac{\partial n_{cell}}{\partial n_{coat}} \cdot \Delta n_{coat}\right)^2 + \left(\frac{\partial n_{cell}}{\partial \lambda_{sca}} \cdot \Delta \lambda_{sca}\right)^2
\] [6.1]
6.2.2 Anti-IGF-1R silver nanospheres

6.2.2.1 Molecular Specificity

Experiments were also performed to demonstrate the molecular specificity of the anti-IGF-1R silver nanosphere conjugation protocol. A549 human alveolar adenocarcinoma cells over-expressing IGF-1R were exposed to the anti-IGF-1R conjugates [60, 105]. Hyperspectral data cubes were acquired for individual cells using the hyperspectral microscopy system previously described in Chapter 3. Scattering spectra averaged over the entire cell were fit to Gaussian distributions, indicating an average peak scattering wavelength of 520.8 ± 13.5 nm for this label (N = 106) (Figure 6.14).

Figure 6.14: Representative image (left) and spectrum (middle) of A549 cell bound with anti-IGF-1R immunolabeled silver nanospheres. (Scale bar = 10 µm)
Figure 6.15: Histogram of peak scattering wavelength of A549 cells bound with anti-IGF-1R 100 nm silver nanospheres. Distribution fit has peak wavelength of 520.8 ± 11.3 nm (N = 102).

Controls consisted of exposure of IGF-1R non-expressing MDA-MB-468 human breast adenocarcinoma cells to anti-IGF-1R nanosphere conjugates [60, 106], IGF-1R expressing A549 cells exposed to IgG1 Ab silver nanospheres, and unexposed A549 cells (Figure 6.16). Control experiments showed negligible increases in scattering, demonstrating statistically significant differences in scattering intensity (<0.001) and molecular specificity (Figure 6.17).
Figure 6.16: Control experiments consisting of (left) IGF-1R expressing A549 cells exposed to IgG Ab nanospheres, (middle) IGF-1R expressing A549 cells not exposed to anti-IGF-1R nanospheres, and (right) IGF-1R non-expressing MDA-MB-468 cells exposed to anti-IGF-1R nanospheres. (Scale bars = 10 µm)

Figure 6.17: Mean scattering intensity of 0.068 ± 0.040 (N= 54) for IGF-1R expressing A549 cells exposed to anti-IGF-1R nanospheres (+/+), 0.019 ± 0.004 (N=50) for A549 cells exposed to IgG Ab nanospheres (+ / IgG), 0.019 ± 0.008 (N= 50) for A549 cells unexposed to conjugates (+ / 0), and 0.020 ± 0.004 (N= 50) for MDA-MB-468 cells exposed to anti-IGF-1R nanospheres (-/+).
6.2.2.2 Refractive index sensing

Determination of the local membrane RI using anti-IGF-1R silver nanospheres requires three separate steps. First, extinction measurements of the uncoated silver spheres suspended in PBS solution are acquired, indicating a peak wavelength of 483.5 ± 0.5 nm. Using the peak extinction and PBS suspension RI of 1.335 as measured using a refractometer (RFM 340, Bellingham Stanley, Kent, UK), Mie theory calculations determine the average nanosphere diameter to be 101.6 ± 0.01 nm, which is in close agreement with the TEM measured value of 99.3 ± 12.0 nm (N=101) [71, 120]. Second, the coated antibody layer thickness of anti-IGF-1R coated gold nanospheres was measured using TEM and determined to be 4.5 ± 1.4 nm (N=80). Using the antibody thickness, in addition to the coated nanosphere extinction peak of 492.3 ± 0.5 nm, coated sphere Mie theory models indicate an antibody layer RI of 1.47 ± 0.04 [120]. Finally, using the peak wavelength of 520.8 ± 11.3 nm (N=102, SEM = 1.12) determined from the Gaussian fit of the distributions of peak scattering from the A549 cells (Figure 6.15), in addition to the derived parameters above, coated sphere Mie theory models determined the local RI at the cell membrane in the volume around the label to be 1.47 ± 0.01 nm. (The sensing distance of the silver nanospheres is approximately 18 nm from the surface, as calculated using a 1/e decay length [20, 121].)
Table 6.6: Refractive index calculation parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>Uncoated Particle Size (TEM)</td>
<td>99.3 ± 12.0 nm</td>
</tr>
<tr>
<td>Antibody Layer Thickness (TEM)</td>
<td>4.5 ± 1.4 nm</td>
</tr>
<tr>
<td>Uncoated Colloid Extinction Peak</td>
<td>483.5 ± 0.03 nm</td>
</tr>
<tr>
<td>Uncoated Particle Size (Extinction)</td>
<td>101.6 ± 0.01 nm</td>
</tr>
<tr>
<td>Coated Colloid Extinction Peak</td>
<td>492.3 ± 0.03 nm</td>
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<tr>
<td>Antibody Layer RI</td>
<td>1.47 ± 0.04</td>
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<tr>
<td>Cell Bound Scatter Peak</td>
<td>520.8 ± 1.12 nm</td>
</tr>
<tr>
<td>Cellular Environment RI</td>
<td>1.47 ± 0.01</td>
</tr>
</tbody>
</table>

6.2.2.3 Uncertainty determination

The uncertainty of the local cellular RI measurement is again determined using the methods outlined by Curry et al. [16, 119]. Calculation of total uncertainty requires the measurement and orthogonal summation of each of the uncertainties associated with the individual parameters. The determination of peak wavelength of cells bound to anti-IGF-1R silver nanospheres has an uncertainty of 1.12 nm, as determined by the standard error of mean of cell scatter peak measurements (SEM). The mean nanosphere radius has an uncertainty of 0.01 nm as determined by the peak extinction uncertainty and the modeled relationship between radius and peak extinction of uncoated colloid. The antibody layer thickness has an uncertainty of 1.49, representing the summation of its respective uncertainties. Finally, the antibody layer RI has an uncertainty of 0.04 RIU, also representing the summation of its respective uncertainties. Each of these uncertainties is then multiplied by their respective differential relationship with the
cellular RI, as seen in Equation 6.1 [16]. From this summation, the total uncertainty is calculated to be 0.01 nm.

6.2.3 Discussion

The RI of biological tissues has been extensively characterized in the literature. Referencing these studies, experimentally determined refractive indices herein provide insight regarding the biological environment of the targeted receptors. Intracellular cytoplasm RI ranges from 1.35-1.38 [116, 122-125], while the cellular membrane RI is higher at a range of 1.46-1.60 [122, 123, 125, 126]. The dielectric environment of the membrane is highly dependent on structural lipids, with an RI of 1.48 [123, 125], and membrane-bound proteins, with an RI of 1.36-1.55 [127]. The RI of 1.47 ± 0.01 determined with the IGF-1R silver spheres and 1.49 ± 0.03 with the anti-EGFR gold nanorods therefore likely indicates proximity to the cell membrane, as expected. The derived RI may also indicate endocytic vesicles comprised of budding cell membrane, which would contain the internalized receptors.

As mentioned previously, a recent work by Curry et al. determined intracellular RI with sub-optical resolution for the first time using a similar technique [16]. In this study, an RI of 1.53 ± 0.02 nm was found for 60 nm gold spheres bound to EGFR. This value, like those determined herein, seems to match the RI expected for proximity with the cell membrane. The slightly higher value seen in Curry’s results may be attributed to
variations in the cellular environment across the different cell lines observed in the respective studies. In this study, the methods established by Curry et al. to execute RI sensing are implemented for the first time using both immunolabeled gold nanorods and silver nanospheres.

Plasmonic coupling represents a potential obstacle to RI sensing using the approach presented. As mentioned previously, coupling between proximal NPs will cause a significant red-shift in peak resonance, preventing accurate modeling of the dielectric environment. Fortunately, relatively lower conjugate concentrations significantly reduce this effect, as seen in Chapter 4. In this study, the concentrations of conjugates produced reasonable dielectric constants which do not indicate that a substantial degree of coupling has occurred. However, in Section 6.3, these coupling effects on RI sensing of the receptor HER-2 while using gold nanosphere labels are observed and addressed.

6.3 Monitoring of receptor dimerization using individual plasmonic labels

In this section, experiments are presented that investigate the conformation of immunolabeled nanospheres bound to membrane receptors undergoing homodimerization. Chapter 5 experiments characterized plasmonic coupling using polarization mapping to assess the ability to sense RI. Based on theoretical simulations, plasmonic coupling effects cannot be entirely separated using polarization mapping.
Therefore, the transverse excitation mode cannot be used to reveal absolute RI without additional \textit{a priori} information regarding the spacing between NP labels, assuming that both proximal receptors have been bound.

Interparticle spacing will depend on the conformation of NPs upon binding to proximal receptors. In this section, the labeling of HER-2 with 60 nm gold nanospheres on SK-BR-3 cells, known for HER-2 overexpression and constitutive dimerization is investigated [46]. The foremost concern in the design of these experiments is that it is unknown whether observation of NP pairs will be possible with the approach presented here. Binding of a 60 nm nanosphere to one receptor may block potential binding of a proximal dimer candidate as originally suspected in Section 4.2.3. In addition, NP concentrations are most likely being implemented below saturation conditions, further reducing the probability of sufficient adjacent receptor labeling.

A previous study has shown that SK-BR-3 HER-2 homodimers have receptor separations of approximately 6.29 nm using FRET techniques [128]. At this spacing, two NP conformations are possible. At 60 nm in size, if both proximal receptors are bound, the two labeled biomarkers would likely come in contact during dimerization, with an interparticle separation dependent on the coated antibody layer thickness. Based on the earlier substrate coupling experiments shown in Section 5.4, this conformation would result in a large, easily distinguishable redshift. A second, less likely conformation would be a greater interparticle distance than the 6.29 nm intermolecular separation.
According to theoretical results shown in Section 5.2, at those distances the total blue-shift due to the orthogonal excitation mode would be a few nanometers, a magnitude that would prevent absolute dielectric sensing, but potentially still allow relative sensing.

RI sensing of a bound receptor dimer requires \textit{in vitro} experiments to address these questions. Scattering spectra of HER-2 overexpressing SK-BR-3 cells bound to HER-2 Ab coated 60 nm gold nanospheres are acquired. The presence of a red-shifted primary peak associated with plasmonic coupling will be shown to confirm the formation of dimers and address concerns regarding proximal receptor blockage and conditions below saturation. Using the RI of the cell membrane environment, as determined by the peak resonant wavelength of scattering by single bound receptors, in conjunction with DDA coupling models, the red-shifted peak wavelength due to plasmonic coupling can be used to determine the NP dimer conformation and spacing.

\section*{6.3.1 HER-2 Ab gold nanospheres}

\subsection*{6.3.1.1 Molecular Specificity}

Experiments were performed to demonstrate the molecular specificity of the HER-2 Ab 60 nm gold nanosphere conjugation protocol. SK-BR-3 human breast adenocarcinoma cells over-expressing HER-2 were exposed to the HER-2 nanosphere conjugates [100, 101]. Because of this high expression level, the HER-2 receptors
constitutively undergo homodimerization. Hyperspectral date cubes were acquired for individual cells using the previously described hyperspectral microscopy system in Chapter 3.

Scattering spectra averaged over the entire cell were fit to Gaussian distributions, indicating an average peak scattering wavelength of $595.1 \pm 26.0$ nm for this label ($N=147$) (Figure 6.18). All peak scattering wavelengths were binned into histograms and fit to a Gaussian distribution. The histogram fit indicated a peak wavelength of $587.0 \pm 11.9$ nm ($N=122$) (Figure 6.19). The histogram did not reveal a second major peak distribution.

Figure 6.18: Representative spectra (left) and image (middle) of SK-BR-3 cell bound with HER-2 Ab immunolabeled 60 nm gold nanospheres. (Scale bar = 10 µm)
Figure 6.19: Histogram of peak scattering wavelength of SK-BR-3 cells bound with HER-2 Ab immunolabeled 60 nm gold nanospheres. Distribution fit has peak wavelength of 587.0 ± 11.9 nm (N = 122).

Controls consisted of exposure of HER-2 non-expressing MDA-MB-468 human breast adenocarcinoma cells to HER-2 Ab nanospheres conjugates [101, 103, 107], HER-2 expressing SK-BR-3 cells exposed to IgG1 Ab gold nanospheres, and unexposed SK-BR-3 cells (Figure 6.20). Control experiments showed negligible increases in scattering, demonstrating statistically significant differences in scattering intensity (p<0.001) and molecular specificity (Figure 6.21).
Figure 6.20: Control experiments consisting of (left) HER-2 expressing SK-BR-3 cells exposed to IgG Ab nanospheres, (middle) HER-2 expressing SK-BR-3 cells not exposed to nanospheres, and (right) HER-2 non-expressing MDA-MB-468 cells exposed to HER-2 nanospheres. (Scale bars = 10 µm)

Figure 6.21: Mean scattering intensity of 0.199 ± 0.137 (N= 147) for HER-2 expressing SK-BR-3 cells exposed to HER-2 Ab nanospheres (+/+), 0.017 ± 0.003 (N= 50) for SK-BR-3 cells exposed to IgG Ab nanospheres (+ / IgG), 0.019 ± 0.003 (N= 50) for SK-BR-3 cells unexposed to conjugates, and 0.017 ± 0.006 (N= 50) for HER-2 non-expressing MDA-MB-468 cells exposed to HER-2 Ab nanospheres (-/+).
6.3.2 Conformation of proximal receptor-bound nanoparticle pairs

First, extinction measurements of the uncoated gold spheres suspended in PBS solution are acquired, indicating a peak wavelength of $535.3 \pm 0.5 \text{ nm}$. Using the peak extinction and known PBS suspension RI of $1.335$, Mie theory calculation determine the average nanosphere diameter to be $59.1 \pm 0.03 \text{ nm}$, which is in close agreement with the TEM measured value of $61.2 \pm 4.3 \text{ nm}$ ($N = 183$)[71, 120]. Second, the antibody layer thickness of HER-2 Ab coated gold nanospheres was measured using TEM and determined to be $5.2 \pm 1.8 \text{ nm}$ ($N = 142$). Using the antibody thickness, in addition to the coated nanosphere extinction peak of $538.7 \pm 0.5 \text{ nm}$, coated sphere Mie theory models indicate an antibody layer RI to be $1.43 \pm 0.03$.

A state of constitutive homodimerization is expected with SK-BR-3 cells. However, only one distinct peak wavelength distribution was observed from 450 - 700 nm. Further investigation is therefore required to determine if the distribution is attributed to scattering by either single spheres or dimer pairs. Using the peak wavelength of $587.0 \pm 11.9 \text{ nm}$ ($N = 122$, SEM = 1.1) determined from the Gaussian fit of the distribution of peak scattering wavelengths from gold nanospheres bound to the SK-BR-3 cells, in addition to the derived parameters above, coated sphere Mie theory models were used to determine the local RI at the cell membrane in the volume around the label to be $1.86$, which is an unrealistically high cellular dielectric constant [120]. Therefore, the peak scattering distribution must also be associated with interactions
among the NPs themselves, potentially indicating plasmonic coupling between HER-2 dimer formation.

Determination of NP conformation and spacing requires a preliminary approximation of local RI using nanospheres bound to individual HER-2 receptors that are not undergoing dimerization. Because there is not a second, smaller peak wavelength distribution, the lowest 5% of peak wavelengths is assumed to be due to bound receptors not undergoing dimerization. These peaks have a mean scattering wavelength of $556.8 \pm 13.1$ nm ($N = 7$). Using this approximation of the mean peak scattering wavelength, a cellular RI of $1.46 \pm 0.07$ is determined, which is in close agreement with the values measured for the anti-EGFR gold nanorod ($1.49 \pm 0.02$) and anti-IGF-1R silver nanosphere ($1.47 \pm 0.01$) labels discussed previously in Sections 6.2.1 and 6.2.2, respectively. RI and corresponding uncertainty are calculated using the methods also described in Section 6.2.2. The large deviation is attributed to the low sample size associated with the lowest 5% of peak scattering wavelengths.

**Table 6.7: Peak scatter calculation parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoated Particle Size (TEM)</td>
<td>$61.2 \pm 4.3$ nm</td>
</tr>
<tr>
<td>Antibody Layer Thickness (TEM)</td>
<td>$5.2 \pm 1.8$ nm</td>
</tr>
<tr>
<td>Uncoated Colloid Extinction Peak</td>
<td>$535.26 \pm 0.03$ nm</td>
</tr>
<tr>
<td>Uncoated Particle Size (Extinction)</td>
<td>$59.06 \pm 0.06$ nm</td>
</tr>
<tr>
<td>Coated Colloid Extinction Peak</td>
<td>$538.69 \pm 0.03$ nm</td>
</tr>
<tr>
<td>Antibody Layer RI</td>
<td>$1.43 \pm 0.03$</td>
</tr>
<tr>
<td>Lowest 5% Cell Bound Scatter Mean</td>
<td>$556.82 \pm 13.09$ nm</td>
</tr>
<tr>
<td>Approximate Cellular RI</td>
<td>$1.46 \pm 0.07$</td>
</tr>
<tr>
<td>Cell Bound Scatter Peak</td>
<td>$587.03 \pm 11.87$ nm</td>
</tr>
<tr>
<td>DDA Model Cell Bound Scatter Peak</td>
<td>$582.1$ nm</td>
</tr>
</tbody>
</table>
A DDA model representing a proximal pair of two touching HER-2 antibody coated 59.1 nm gold nanospheres, each consisting of a distinct core and mantle dielectric constant, is established using the information gained from these steps. The antibody layer has a RI of $1.43 \pm 0.03$, while the spheres are simulated in a surrounding RI of $1.46 \pm 0.07$, representing the approximation of the dielectric environment of the receptor-bound particles on the cell membrane. The interparticle separation of the two gold cores in the model consists solely of the two 5.2 nm antibody layer thicknesses, for a total separation of 10.4 nm. The simulation indicates a peak wavelength of 582.1 nm, which is in close agreement with the distribution peak of $587.1 \pm 11.9$ nm (N=122) observed in the HER-2 Ab labeling cell experiment. Models were also computed for the same two gold nanospheres with the standard deviations of coating thickness, 3.4 and 7.0 nm. In these cases, the peak scattering is determined to be 592.9 and 570.9 nm, respectively. Recall from Chapter 5 that the plasmonic coupling redshift of a particle pair decays exponentially with decreased interparticle separation. These results indicate interparticle distances are at or slightly below double the mean of the antibody layer thickness, confirming a conformation in which the particles are touching.

### 6.3.3 Discussion

In cell lines that express high levels of HER-2, such as SK-BR-3 adenocarcinoma cells, the receptors are known to undergo constitutive homodimerization [46]. In fact,
the conformation state of the receptor favors dimerization both with itself and other
ErbB family members [46]. HER-2 antibody activation further accelerates this
homodimerization and consequential endocytosis of the receptors [129]. Evaluation of
dimerization by FRET methods also confirms homodimer formation, specifically in SK-
BR-3 cells [130].

Several implications can be realized from the understanding of the NP dimer
conformation observed in the presented experiments with SK-BR-3 cells. First, scattering
spectra indicate proximal nanospheres are touching and the distances between gold
cores are dependent on the coated antibody layer thickness. Second, larger nanospheres
on the order of 60 nm are not appropriate FRET alternatives for the monitoring of HER-
2 spacing, which is on the order of 6.29 nm [128]. Because models indicate the proximal
labels are in contact upon receptor binding, the longitudinal excitation mode does not
provide intermolecular distance information. Finally, scattering peak wavelengths that
are further red-shifted from those predicted for the dimer peak resonance likely indicate
the formation of higher order oligomers or endocytic vesicle uptake [131].

Plasmonic NPs have recently been used for tracking of intermolecular
separations between dimers of fibronectin-integrin complexes on HeLa cell membranes
[132]. As the complexes underwent compaction, a change in distance between complex-
bound 40 nm gold nanospheres was observed from 15 to 6 nm, with 6 nm representing
the thickness of the PEG and anti-fibronectin coating layer. Plasmonic tracking of
receptor spacing has several advantages over current FRET techniques, which are limited by photobleaching, auto-fluorescence, and spectral bleed-through [3]. More importantly, plasmonic NPs can be used for measurements above the limit of FRET (typically 10 nm [133]), but still under the diffraction limit of optical microscopy [134]. However, the experiments detailed herein demonstrate for the first time the potential of cellular RI sensing by the NP pair.

While RI sensing of the local dimer environment with a single bound NP is likely possible at lower conjugate concentrations, removing the need for a priori interparticle distance information, an important advantage of using NP pairs would be lost, plasmonic coupling enhancement of dielectric sensing. Sensing ability of bound ligand by a NP is measured by the extent of the localized surface plasmon resonance (LSPR) shift divided by the change in RI. Several methods exist for enhancing sensitivity, with usual emphasis on increasing the particle size to mass ratio [92]. Theoretical simulations by Jain et al. have demonstrated that coupling between two gold 40 nm nanospheres provides an exponential increase in medium sensitivity as interparticle distance decreases [93]. The enhancement was experimentally demonstrated in NP sensor arrays that increased dielectric sensitivity three-fold [94]. Simulations in Section 5.2.2 further demonstrated this enhanced sensing effect with the same 60 nm gold nanospheres implemented in the study herein.
The results also exhibit peak scattering at wavelengths even longer than those indicative of NP dimer pairs. Due to the upper range limitations of our hyperspectral imaging system (700 nm), the implications of this scattering were not fully explored. However, the existence of scattering peaks at such long wavelengths likely indicates the presence of HER-2 clusters consisting of higher order oligomers or endocytic vesicle uptake. SK-BR-3 cells express such clusters, approximately 500 nm in diameter, with upwards of 1000 HER-2 proteins each, as found through scanning near field optical microscopy [135]. A previous study has applied anti-EGFR immunolabeled plasmonic NPs to the monitoring of receptor regulation state [131]. As receptors undergo endocytosis from cell membrane to early endocytic vesicles, and eventually towards multi-vesicular bodies, there is a red shift in peak wavelength associated with plasmonic coupling to higher order aggregates of the receptor bound NPs. The experimentally determined peak wavelengths correlate well with theoretical values found using electrodynamic modeling.

In Chapter 5, theoretical simulations indicated a need for interparticle spacing information in order to measure absolute RI. Experiments with SK-BR-3 cells shown above, revealed dimer-bound interparticle distances solely dependent on coating thickness and not intermolecular spacing. This conformation is likely due to the large nature of the labels. The inability to entirely separate plasmonic effects in addition to a simpler method of determining interparticle spacing potentially negates the need for
polarization mapping. Nonetheless, coupling provides the additional advantage of enhanced dielectric sensing, as demonstrated in Chapter 5. Polarization mapping could still be exploited to isolate the longitudinal excitation mode, which primarily exhibits the enhanced sensing ability. The technique would be especially beneficial at slightly larger interparticle distances where the primary and secondary scattering peaks overlap and require polarization mapping to accurately determine the primary peak resonance.

6.4 Multiple simultaneous plasmonic labels

6.4.1 Multiplex molecular imaging

Multiplexed molecular imaging plays an important role in gaining a better understanding of cellular signaling pathways. Understanding of these interactions has led to the development of new drugs and therapies for the treatment of multiple diseases, including cancer. Molecular imaging has applications for in vivo imaging as well. Early stage cancer cells overexpress particular receptors that can be monitored to indicate immunophenotype and therefore metastatic potential, prior to more identifiable changes in internal cellular structure, size, or shape. Because tumorigenesis involves multiple signal pathways, multiplexing of molecular imaging information can improve the accuracy of early diagnosis [4]. Furthermore, clinical studies have shown that certain immunophenotypes confer resistance to some cancer drugs and therefore provide an indication of the best course of disease therapy. In fact, one heterogeneous malignant
tumor may contain multiple cell types with varying responsiveness to drug therapy [136]. Successful resection of a malignant tumor also benefits from molecular imaging. Without well-defined borders of the cancer cells invading healthy tissue, surgeons must rely on some method of differentiating the malignancy. Certain contrast agents, such as carbon nanotubes also confer therapeutic properties specific to the malignant tissue that increases in efficiency when multiplexed [6]. For these reasons, multiplexed molecular imaging has found applications in optical imaging, MRI, ultrasound, and PET.

While multiplexing confers these benefits, the spectral extent of available fluorescent tags is still primarily limited to visible wavelengths. This limitation makes multiplex labeling above seven simultaneous tags difficult to achieve. Quantum dots, the near-infrared alternative to fluorescence, provide a potential method to further increase the extent of multiplexing by taking advantage of a different spectral range. However, the application of quantum dots is limited by its acute cytotoxicity properties, although current advances in coating and encapsulation are reducing these effects [7, 8]. A potential alternative class of biomarkers is plasmonic NPs. Variations in shape, size, and composition greatly affect the peak scattering of these NPs, enabling access to various spectral regions via simple modifications in synthesis. For instance, gold nanorods can easily be fabricated with various length-to-width aspect ratios, providing scattering from 600 up to 2200 nm. This extended scattering wavelength range by itself would greatly expand the degree of multiplexing available, while avoiding cytotoxicity
concerns. Other NP types provide peak scattering in alternative spectral windows with gold spheres covering 500 - 600 nm, and silver spheres from 400- 500 nm, for example.

6.4.2 EGFR and HER-2

Experiments were performed to demonstrate relative immunophenotyping using two functionalized NPs in cells with established EGFR / HER-2 receptor expression levels (Figure 6.22). In each case, cell lines were exposed to anti-EGFR gold nanorods first, followed by HER-2 Ab nanospheres. EGFR(+)/HER-2(-) MDA-MB-468 cells revealed primarily one peak associated with scattering from the long axis of the anti-EGFR coated nanorod [60, 99-101, 103, 107]. EGFR(-)/HER-2(+) MDA-MB-453 cells demonstrated only a single peak associated with the HER-2 Ab coated nanospheres [60, 100, 101]. The HER-2 label additionally exhibited low amounts of red-shifted scatter. This larger distribution is likely attributed to low levels of plasmonic coupling, as seen previously in Section 6.4 with HER-2 in SK-BR-3 cells. Nonetheless, the distribution does not overlap with peak scattering observed with the anti-EGFR nanorods. EGFR(-)/HER(-) COLO-320DM cells revealed no significant binding [100].
Additional experiments further confirmed the ability to perform relative immunophenotyping with dual labeling. First, A549 / HER-2 cells were individually labeled with either anti-EGFR gold nanorods or HER-2 Ab gold nanospheres. The anti-EGFR label exhibited a peak scattering wavelength of 656.1 ± 9.5 nm (N=73), while the HER-2 Ab label exhibited a peak of 542.7 ± 21.3 nm (N=75) (Figure 6.23). These two peak distributions were statistically distinct with a p value of <0.001, indicating statistically significant differences in scattering behavior, as expected.
Figure 6.23: Distribution of peak scattering from A549 / HER-2 cells individually exposed to either anti-EGFR nanorods (left) or HER-2 Ab nanospheres (right).

Next, these same cells were labeled consecutively with anti-EGFR gold nanorods and HER-2 Ab gold nanospheres (Figure 6.24). The anti-EGFR label exhibited a peak scattering wavelength of $657.8 \pm 15.8$ (N=132), while the HER-2 Ab label exhibited a peak of $551.4 \pm 8.6$ (N=35) (Figure 6.25). These two peak distributions were analyzed and found to exhibit statistically significant differences (p<0.001) in scattering behavior when labeling the same cell simultaneously.

Table 6.8: Measured Parameters of EGFR and HER-2 Dual Label Experiments

<table>
<thead>
<tr>
<th>EGFR and HER-2 Trials</th>
<th>Peak (nm)</th>
<th>N</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-468</td>
<td>663.7 ± 6.6</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>586.9 ± 24.1</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td><strong>A549 / HER-2 Individual Exposure</strong></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EGFR</td>
<td>656.1 ± 9.5</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>HER-2</td>
<td>542.7 ± 21.3</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td><strong>A549 / HER-2 Dual Exposure</strong></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EGFR</td>
<td>$657.8 \pm 15.8$</td>
<td>132</td>
<td>0.724</td>
</tr>
<tr>
<td>HER-2</td>
<td>$551.4 \pm 8.6$</td>
<td>35</td>
<td>0.045</td>
</tr>
</tbody>
</table>
The peak scattering distributions of the individually labeled cells are then compared to those of the simultaneously dual labeled cells. A p-value = 0.724 is determined for the anti-EGFR label associated scattering compared to the same conjugate in the single label measurements. A p-value = 0.045 is determined for the HER-2 Ab label associated scattering compared to the single label measurements of the same label. However, this p-value increases to 0.145 when excluding values that do not correspond with either peak distribution, such as the smaller peak seen around 600 nm in Figure 6.25.

Figure 6.24: Representative spectra (left) and image (right) of A549 / HER-2 cell exposed to anti-EGFR nanorods and HER-2 Ab gold nanospheres consecutively. (Scale bar = 10 µm)
6.4.3 EGFR and IGF-1R

Experiments were performed to demonstrate relative immunophenotyping using two functionalized NPs in cells with established EGFR / IGF-1R expression levels (Figure 6.26). In each case cell lines were exposed to anti-EGFR nanorods first, followed by anti-IGF-1R nanospheres. EGFR(+)/IGF-1R(-) MDA-MB-468 cells revealed primarily one peak associated with scattering from the long axis of the anti-EGFR coated nanorod [60, 99, 100, 106]. EGFR(-)/IGF-1R(+) MDA-MB-453 cells demonstrated only a single peak associated with the anti-IGF-1R coated nanospheres [60, 100]. Both cases exhibited low levels of peak scattering for the negative receptor label.
Additional experiments further confirmed the ability to perform relative immunophenotyping with dual labeling. First, A549 cells were individually labeled with either anti-EGFR gold nanorods or anti-IGF-1R silver nanospheres. The anti-EGFR label exhibited a peak scattering wavelength of 648.4 ± 14.5 nm (N=73), while the anti-IGF-1R label exhibited a peak of 520.5 ± 11.6 nm (N=75) (Figure 6.27). These two peak distributions present statistically significant differences with a p value of <0.001, indicating distinct scattering behavior, as expected.
Next, these same cells were labeled consecutively with the anti-EGFR gold nanorods and anti-IGF-1R silver nanospheres (Figure 6.28). The anti-EGFR label exhibited a peak scattering wavelength of 658.3 ± 17.6 nm (N = 102), while the anti-IGF-1R label exhibited a peak of 533.1 ± 15.2 nm (N = 81) (Figure 6.29). These two peak distributions reveal statistically significant differences (p<0.001) in scattering behavior when labeling the same cell simultaneously.

Figure 6.28: Representative spectra (left) and image (right) of A549 cell exposed to anti-EGFR nanorods and anti-IGF-1R nanospheres consecutively. (Scale bar = 10 µm)
Figure 6.29: Distribution of peak scattering from A549 cells exposed to anti-EGFR gold nanorods and anti-IGF-1R nanospheres consecutively.

The peak scattering distributions of the individually labeled cells are then compared to that of the simultaneously dual labeled cells for each label. A p-value of <0.001 is determined for both the anti-EGFR and anti-IGF-1R label associated scattering.

Table 6.9: Measured Parameters of EGFR and IGF-1R Dual Label Experiments

<table>
<thead>
<tr>
<th>EGFR and IGF-1R Trials</th>
<th>Peak (nm)</th>
<th>N</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-468</td>
<td>669.9 ± 11.2</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>517.4 ± 8.5</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>A549 Individual Exposure</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EGFR</td>
<td>648.4 ± 14.5</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>HER-2</td>
<td>520.5 ± 11.6</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>A549 Dual Exposure</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EGFR</td>
<td>658.3 ± 17.6</td>
<td>102</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HER-2</td>
<td>533.1 ± 15.2</td>
<td>81</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Finally, these same cells were labeled consecutively in the reverse order with the anti-IGF-1R silver nanosphere incubation first, followed by the anti-EGFR gold nanorods. The anti-IGF-1R label exhibited a peak of 523.1 ± 6.9 nm (N=46), while the EGFR label exhibited a peak scattering wavelength of 643.0 ± 5.3 nm (N=14) (Figure 6.30). These two peak distributions reveal a p value of <0.001, indicating statistically significant differences in scattering behavior when labeling the same cell simultaneously.

Figure 6.30: Distribution of peak scattering from A549 cells exposed to anti-IGF-1R nanospheres and anti-EGFR gold nanorods consecutively.

More interestingly however, is the comparison between the peak scattering distributions of the individually labeled cells and that of the simultaneously dual labeled cells in reverse order. Foremost, a large reduction in anti-EGFR gold nanorod binding and scatter is observed with this labeling sequence. Also, unlike the original order, a p-
value = 0.759 is determined for the anti-IGF-1R label associated scattering compared to that found for the single label, and a p-value = 0.201 is determined for the anti-EGFR label associated scattering, also compared to that found for the single label. The lack of statistical significance in either receptor case indicates no detectable change in dielectric environment when labeled in reverse order.

### 6.4.4 IGF-1R and HER-2

<table>
<thead>
<tr>
<th>IGF-1R and HER-2 Trials</th>
<th>Peak (nm)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>520.4 ± 11.0</td>
<td>49</td>
</tr>
<tr>
<td>BT-474</td>
<td>566.8 ± 15.8</td>
<td>49</td>
</tr>
</tbody>
</table>

Experiments were also performed to demonstrate relative immunophenotyping of two functionalized NPs in cells with established IGF-1R / HER-2 receptor expression levels (Figure 6.31). In each case cell lines were exposed to anti-IGF-1R nanorods first, followed by HER-2 Ab nanospheres. IGF-1R(+)/HER-2(-) MCF-7 cells revealed primarily one peak associated with scattering from the anti-IGF-1R coated nanospheres [60, 102, 103]. IGF-1R(-)/HER-2(+) BT-474 cells demonstrated only a single peak associated with the HER-2 Ab coated nanospheres [60, 102, 103]. In the case of BT-474, according to the literature the negative receptor (IGF-1R) is technically expressed, as seen in ELISA
results in Table 6.9, but at lower levels relative to the positive receptor (HER-2) which is significantly over-expressed, which is also confirmed by ELISA. IGF-1R(-)/HER(-) MDA-MB-468 cells revealed no significant binding [60, 101, 103, 106, 107].

![Figure 6.31](image)

**Figure 6.31:** Distribution of peak scattering from (left) IGF-1R(+)/HER-2(-) MCF-7 cells and (right) IGF-1R(-)/HER-2(+) BT-474 cells exposed to anti-IGF-1R nanospheres and HER-2 Ab gold nanospheres consecutively.

### 6.4.5 EGFR, IGF-1R, and HER-2

The final experiments in this section seek to establish the ability to perform relative immunophenotyping with triple labeling. First, the A549 / HER-2 cells were individually labeled with either anti-EGFR gold nanorods, anti-IGF-1R silver nanospheres, or HER-2 Ab gold nanospheres to demonstrate the presence of the three targeted receptors. Upon individual label exposure and cell binding, a peak scattering wavelength of $656.1 \pm 9.5 \text{ nm (N}=73)$ is observed for the anti-EGFR case, $521.8 \pm 7.9 \text{ nm}$
(N=75) for the anti-IGF-1R case, and 542.7 ± 21.3 nm (N=75) for the HER-2 Ab case. Each of the pairings of these two peak distributions are statistically distinct with a p value of <0.001, indicating statistically significant differences in scattering behavior, as expected and confirming that these three receptors were present.

![Figure 6.32](image)

**Figure 6.32: Distribution of peak scattering from A549 / HER-2 cells individually exposed to either anti-EGFR nanorods (left), HER-2 Ab nanospheres (middle), or anti-IGF-1R nanospheres (right).**

Next, these same cells were labeled consecutively with anti-EGFR nanorods, anti-IGF-1R nanospheres, and HER-2 Ab nanospheres (Figure 6.33). The anti-EGFR label exhibited a peak scattering wavelength of 661.3 ± 12.7 (N=127), while the HER-2 Ab label exhibited a peak of 555.8 ± 7.2 (N=44) (Figure 6.34). There was no discernible peak associated with the anti-IGF-1R label. These two peak distributions are distinct with a p value of <0.001, indicating statistically significant differences in scattering behavior when labeling the same cell simultaneously. The lack of detected IGF-1R binding is explained by the relatively high degree of HER-2 expression. The HER-2 Ab nanosphere
scattering is likely masking the scattering from the anti-IGF-1R silver nanospheres. However, a few cells did exhibit high enough expression of IGF-1R to be detected in the average cell spectra, as seen in Figure 6.28.

Table 6.11: Measured Parameters of EGFR, IGF-1R and HER-2 Triple Label Experiments

<table>
<thead>
<tr>
<th>EGFR, IGF-1R, and HER-2 Trials</th>
<th>Peak (nm)</th>
<th>N</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549 / HER-2 Individual Exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>656.1 ± 9.5</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>IGF-1R</td>
<td>521.8 ± 7.9</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>HER-2</td>
<td>542.7 ± 21.3</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>A549 / HER-2 Triple Exposure</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EGFR</td>
<td>661.3 ± 12.7</td>
<td>127</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>HER-2</td>
<td>555.8 ± 7.2</td>
<td>44</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Figure 6.33: Representative spectra (left) and image (right) of A549 / HER-2 cell exposed to anti-EGFR nanorods, anti-IGF-1R nanospheres, and HER-2 Ab nanospheres consecutively. (Scale bar = 10 µm)
Figure 6.34: Distribution of peak scattering from A549 / HER-2 cells exposed to anti-EGFR nanorods, anti-IGF-1R nanospheres, and HER-2 Ab nanospheres consecutively.

Figure 6.35: Representative spectra (left) and image (right) of A549 / HER-2 cell exposed to anti-EGFR nanorods, anti-IGF-1R nanospheres, and HER-2 Ab nanospheres consecutively. (Scale bar = 10 µm)
The peak scattering distributions of the individually labeled cells are compared to those of the simultaneously dual labeled cells. A p-value of <0.001 is determined for both the anti-EGFR and HER-2 Ab label associated scattering. These results are particularly interesting given the lack of statistical significance observed in the EGFR / HER-2 dual and single labeling comparison experiments. The IGF-1R label potentially caused a change in the interaction between the three receptors resulting in significantly different cellular refractive indices.

6.4.6 Discussion

In this section, a novel tool for simultaneous investigation of multiple receptors has been presented. Previous multiple tag experiments using plasmonic labels primarily looked at nanorod labels of various length to width ratios to produce scattering peaks in different spectral windows [37]. In the work presented here, an alternative approach has been demonstrated that utilizes labels of different size and composition to take advantage of further visible scattering windows. Three receptors commonly overexpressed in cancer cells have been targeted, allowing for immunophenotype profiling applications. Because the NP technique does not suffer from photobleaching or cytotoxicity, it represents an advantageous alternative to fluorescence multiplexing.

The work presented here has foremost shown the ability to determine relative cell immunophenotype of two receptors through dual labeling validation experiments.
on cells with known receptor expression levels. In these cells, one receptor is significantly over-expressed, while the other is relatively low or non-expressing. All cell lines implemented are well characterized in the literature, but ELISA screening provides additional confirmation of expression profile. Both EGFR / HER-2 and EGFR / IGF-1R experiments used cells with clear positive / negative expression levels. However, in the EGFR / IGF-1R experiments small peak scattering distributions over the cell samples were observed, as seen in Figure 6.26 at approximately 525 nm (EGFR(-) / IGF-1R(+)) case, left figure) and 650 nm (EGFR(+) / HER-2(-) case, right figure). This minimal level of scattering is attributed to non-specific binding. In addition, the IGF-1R / HER-2 experiments used MCF-7 and BT-474 cells with distinct overexpression of IGF-1R and HER-2, respectively. ELISA results however indicate some expression of the “negative” receptor as well, despite only one observed scattering distribution corresponding to each of the positive receptors, as seen in Figure 6.31. This behavior is expected given ELISA results indicating expression of the “negative” receptor at significantly lower levels relative to the “positive” receptor.

Additional experiments were conducted exposing cells expressing closer relative receptor levels with two and three functionalized NPs, each targeted to one of the receptors. Controls were performed on the same cell lines with exposure to only one of the conjugates. The comparison between each of the individual label exposures exhibited statistically significant differences in peak scattering distributions for both the
EGFR / IGF-1R and EGFR / HER-2 cases. Such differences indicate adequate separation between the two labels. This is further confirmed by the total shift between primary peaks, which is over 125 nm for EGFR / IGF-1R and over 110 nm for EGFR / HER-2. Such distinct scattering profiles allow for easy identification by eye of various pockets of receptor expression. This behavior would have benefits in applications requiring multiple biomarkers with visible qualitative differences in peak scattering. One in vivo application would be the identification of malignant tumor margins within healthy tissue for phenotypes known to express these particular permutations of receptors, providing an additional level of specificity.

In some cases, labels targeted for a particular receptor in a specific cell population exhibit statistically significant differences in scattering peaks between individual and dual labeling experiments. This behavior is seen in the EGFR / IGF-1R dual label (original order) and the EGFR / IGF-1R / HER-2 triple label experiments. These findings may potentially indicate that slight variations in the cellular dielectric environments have been introduced due to exposure of the second label. These changes could be the result of anything from a change in cellular solute concentration, to cross-talk between the two activated receptors, including heterodimer formation or transphosphorylation. Receptor interaction provides an additional possibility that a degree of plasmonic coupling between the receptor-bound NPs themselves is causing the observed red shift. More interestingly, in the triple label experiment, a change in
behavior is observed from the dual labeling of the same cell population, indicating the effects of the additional IGF-1R conjugate. Because the methods may provide information regarding dielectric environment changes, the technique represents an additional approach to observation of cellular signaling pathways, but requires further investigation to become a useful tool for this application. Polarization mapping represents one potential tool to help discern the dielectric changes due to cross-talk between receptors and cross-talk between coupling labels.

A lack of statistical significance between peak scattering of particular functionalized NPs implemented individually and in conjunction with a second label is observed in two trials. This behavior is first exhibited in the EGFR / HER-2 labeling of A549 / HER-2 cells with anti-EGFR gold nanorods (p = 0.724), and the HER-2 Ab gold nanospheres (p = 0.145, excluding peak values that do not correspond with either peak distribution). The absence of a change in peak scattering indicates little change in the dielectric environment around the particles, which includes plasmonic coupling effects of proximal particles. Additionally, comparison of the HER-2 Ab label scatter also presents evidence that the dual label experiment was not simply showing two bimodal scattering peaks associated with each of the nanorod axes. Rather, similar primary scattering peaks are observed in both individual and dual label cases, likely corresponding with HER-2 in both cases. The borderline statistical significance observed in the non-excluded HER-2 peak comparison (p = 0.045) is likely due to plasmonic
coupling, but may also be attributed to overlap with the anti-EGFR gold nanorod primary peak scatter. A similar absence of change in peak scattering is also exhibited for the IGF-1R / EGFR reverse order trial.

The data presents evidence of a degree of overlap between the peak resonance bands of the HER-2 Ab gold nanosphere and anti-IGF-1R silver nanosphere labels. For instance, in the simultaneous triple label experiment a distribution peak associated with anti-IGF-1R scatter is not observed across the cell samples, despite being present in individual anti-IGF-1R labeling of the same cells. Individual exposures indicate a peak resonance of 542.7 ± 21.3 for HER-2 Ab conjugates and 512.0 ± 13.2 for anti-IGF-1R conjugates, representing a total shift of approximately 30 nm. In retrospect, the silver nanosphere size should have been chosen at a lower peak resonant wavelength, but availability limited smaller particle options. The low expression levels of IGF-1R in these cells, and the low binding efficiency of the label further exasperates the problem. As mentioned previously, the same issue between HER-2 Ab and anti-IGF-1R labels occurs in the BT-474 dual label control. While BT-474 is technically an IGF-1R expresser, the quantity of HER-2 is so large that no peaks associated with IGF-1R are detected in the distribution of peaks for all cell samples.

Overlap between the off resonance the anti-IGF-1R and HER-2 Ab conjugates prevents detection of lower levels of IGF-1R. For this reason, future experiments should aim to narrow the spectral peaks associated with each tag and reduce such overlap.
Several avenues are available to ensure such characteristics. First, the distribution in size can be reduced through improved fabrication techniques. Second, novel shapes (i.e., stellated) and dielectric conformations (i.e., nanoshells) can fine-tune the peak resonance bandwidth for various applications. Third, at lower functionalized NP concentrations, there is a reduction in plasmonic coupling-dependent peak shifts known to broaden the average bandwidth, as discussed in Chapter 4. Finally, as mentioned above, the secondary peak associated with the transverse mode of nanorods does not appear to change the scattering profile of the other labels in these trials, but remains a concern. This can easily be addressed by using only nanorods of similar width, but increasing length. Such conformations would result in nearly identical secondary peaks but increasingly shifted primary peaks associated with the longitudinal scattering mode. Nonetheless, NPs of all shapes and sizes will have some extent of off-resonance scatter. Reliance on peak shift for observation of the presence of a particular receptor may require a more appropriate method to account for these effects.

As mentioned in Section 2.4.2, “lab in a tube” biosensors have been explored recently [35]. These assays consist of a colloid suspension containing three distinct NPs functionalized to different antigens. Upon ligand binding, an observable shift is exhibited on the respective NP spectra. Previous work is somewhat limited, but some of the results address the issues encountered in our work. For instance, such a setup will not suffer from a change in particle concentration upon receptor binding. Therefore, if
conjugates are chosen appropriately, low receptor expression will not result in masking by the over-expressed receptor. The labels and cell lines studied here can easily be applied towards this technique, simply by collecting and preparing the necessary membrane protein.

Reverse order dual labeling in the EGFR/IGF-1R experiment exhibited an interesting change in scattering behavior relative to the original order trial. Rather than observing similar scattering behavior in each case, the reverse order showed a drastic decrease in the amount of anti-EGFR gold nanorods binding following incubation with anti-IGF-1R silver nanospheres. Experiments were performed on cells plated simultaneously and incubated with conjugates from identical batches. First impressions would indicate some potential blocking of the gold nanorods by the relatively larger silver nanospheres. However, images show a distinct difference in the number of NPs bound to the cell in each individual labeling case. The total number of silver nanospheres usually remains under twenty, indicating low binding efficiency, and more importantly the inability to adequately block a significant amount of EGFR. A study by Desbois-Mouthon et al. offers potential insight into this behavior [61]. Activation of IGF-1R induces the release of the EGF-ligand, amphiregulin, which leads to EGFR activation in hepatoma cells. A similar release may be occurring in these alveolar adenocarcinoma cells, binding a significant amount of EGFR prior to incubation with the anti-EGFR
nanorods. Further investigation outside of the scope of this work is required to fully understand the behavior.

The immunophenotype profiling results do not provide receptor expression distribution information over the cell population. For instance, only cells with an observable amount of label scattering were measured for these experiments, potentially giving the false impression of expression over the entire population. This additional knowledge would give a more clear indication of true immunophenotype. The standard method of profiling such distributions is flow cytometry. In the next chapter (Chapter 7), experiments are conducted to demonstrate profile analysis using anti-EGFR gold nanorods. Successful implementation of these particles for the first time may provide an avenue for measuring receptor distribution with multiple plasmonic NPs over the cell population.

In conclusion, results have shown the use of three functionalized plasmonic NP conjugates for simultaneous molecular imaging of multiple receptors. Validation experiments of dual labeled cell lines demonstrate the ability to identify relative expression levels of two receptor types. More importantly, changes in the dielectric environment are detected between single and dual labeling experiments, indicating either cross talk between receptors affecting the cellular environment or plasmonic coupling between the labels themselves. This technique provides an additional tool in the investigation of cellular receptor interactions. Further research is required to discern
the exact cause of these dielectric changes. Fortunately, polarization mapping, previously explored in Chapter 5, offers one approach to identify the source of these shifts in RI. Additional improvements to these methods include increased separation between the chosen labels, or the application of the developed conjugates toward alternative biosensor configurations.

6.5 Conclusion

In this chapter, simultaneous multiplex detection of three different classes of plasmonic NPs has been demonstrated, with each NP type targeted to a receptor commonly overexpressed in metastatic cancers, including anti-EGFR gold nanorods, anti-IGF-1R silver nanospheres, and HER-2 Ab gold nanospheres. The study also represents the first time in which either anti-IGF-1R has been conjugated to silver nanospheres, or HER-2 Ab to gold nanospheres. The extension of the approach to include these various NP types is significant as each label scatters strongly in a separate spectral window. The ability to monitor both of these receptors simultaneously will enable new experiments to investigate the mechanisms in which these different receptor signaling pathways interact or potentially open up new avenues of photothermal therapy. Second, the molecular specificity of all three tags has been established. Third, using a combination of Mie theory and DDA, the local RI of the cellular membrane has
been determined for each cell type. This information can potentially provide a means to understand mechanisms of receptor internalization after binding. In the case of HER-2 receptors, the conformation of proximal NPs bound to receptors undergoing dimerization is also investigated. Finally, simultaneous dual and triple tag experiments demonstrated the ability to profile the immunophenotype of various cell lines with dielectric sensing of receptor interactions in the cellular environment.
7 Flow cytometry using plasmonic nanoparticles

In this chapter, plasmonic NP-based flow cytometry is investigated [137]. Fluorescence-based flow cytometry measures multiple cellular characteristics, including levels of receptor expression, by assessing the fluorescence intensity from a population of cells whose cell surface receptors are bound by a fluorescently labeled antibody or ligand for that receptor. Functionalized noble metal NPs provide a complementary method of receptor labeling based on plasmonics for population analysis by flow cytometry. The potential benefits of using plasmonic NPs to label cell surface receptors in flow cytometry include scattering intensity from a single particle that is equivalent to fluorescence intensity of $10^5$ fluorescein molecules, biocompatibility and low cytotoxicity, and non-quenching optical properties. The large spectral tunability of nanorods also provides convenient access to plasmonic markers with peak surface plasmon resonances ranging from 600-2200 nm, unlike gold nanosphere markers that are limited to visible wavelengths. Gold nanorod-based plasmonic flow cytometry is demonstrated herein by comparing the scattering of cells bound to anti-EGFR conjugated nanorods to the emission of cells bound to anti-EGFR-conjugated fluorescent labels. EGFR expressing cells exhibited a statistically significant, six-fold increase in scattering when labeled with anti-EGFR conjugated nanorods compared to labeling with IgG1 conjugated nanorods. Large scattering intensities were observed despite employing
a thousand fold lower concentration of nanorod conjugated antibody relative to the fluorescently labeled antibody.

Flow cytometry provides a quick and easy analysis of large populations of cells, including measurements of size, granularity, and fluorescence intensity [64]. Flow cytometers can also be equipped for FACS, which discriminates the cells based on the population properties. The basics of flow cytometry involve laser illumination of a stream of particles, which can include cells. The incident light scatters from the particles or causes excitation of fluorescent labels. The scattered and emitted light is detected and interpreted into a histogram of the population cellular properties. For example, forward scattered light corresponds with cell size, while side scattered light corresponds with cell granularity and internal cellular structures. Incident light also excites any fluorophore tags bound to the cell membrane. The emitted fluorescence is collected and delivered to multiple filter channels specific to the fluorescent tags [138]. The intensity recorded corresponds to a relative expression level of the receptor of interest.

### 7.1 Nanoparticle based flow cytometry

Multiplexed fluorescent labeling is currently the most widely used method for immunophenotype profiling of cell populations in flow cytometry, but some limitations on their use still remain. Commercially available fluorescent labels typically emit over a narrow spectral range, predominantly in the visible region, making it difficult to
perform multiplex labeling with more than seven tags simultaneously [37, 139]. The largest multi-parametric analysis performed to date included seventeen individual tags comprised of fluorochromes and quantum dots with a maximum emission of 705 nm [140].

Gold nanorods may be a suitable alternative contrast agent for immunophenotype profiling using flow cytometry. While gold nanospheres are limited to visible wavelengths (due to increased spectral width at larger diameters), gold nanorods can easily be fabricated with various length-to-width aspect ratios, corresponding to peak scattering wavelengths ranging from 600 to 2200 nm, which is outside the spectral range of scattering exhibited by cellular structures [37, 141, 142]. This broad spectral range potentially allows for up to 20 nanorod tags to be used simultaneously in flow cytometry (assuming an 80 nm spectral absorbance width per tag) by incorporating a broadband light source into the setup [37]. This broad spectral range could be used to provide a potential doubling of the number of different receptors that could be monitored, as compared to conventional fluorescent labels.

Gold nanospheres have previously been explored previously for use in flow cytometry but with limited success. Previous attempts investigated the use of 40 nm gold nanospheres immunolabeled with anti-mouse IgG. The flow cytometer in these experiments used a He-Ne source with an excitation wavelength of 632.8 nm and detected the scattered light from the NPs at 90° from the excitation beam. While the
results showed a ten-fold enhancement in signal intensity over unlabeled cells, the small scattering cross-section associated with this NP, due to the poor mismatch between the excitation source and peak resonance wavelength of the NP, significantly limited the measured scattering intensity [143, 144]. Plasmonic coupling between nanosphere clusters most likely accounted for the small, detected enhancement, but required a significant increase in nanosphere concentration to observe the increased signal. Smaller immunolabeled gold nanospheres of 1 and 30 nm diameters were also investigated as scattering labels in flow cytometry using a 488 nm excitation source [145]. For similar reasons, these experiments with labeled lymphocytes did not show a difference in light scattering between receptor-positive cells and controls. The immunolabeled cells demonstrated a clear difference between receptor-positive cells and controls only through silver enhancement of the NPs bound to the cell membrane. Gold NPs have also been used with fluorescent tags for flow cytometry, which allowed multiplexed measurements using both labels [143, 146].

Further work demonstrated the use of gold and silver nanosphere-coated 2 µm polystyrene beads for immunophenotyping [147]. This study appears to be the first that considered using an excitation source that matched the Mie theory-predicted surface plasmon resonance of the NPs. While this approach was seen to increase contrast when compared to binding of immunolabeled NPs directly to the cell membrane, the method additionally alters the shape of the cell, allowing for an alternative method of
differentiation, as opposed to their detection by solely exploiting the plasmonic behavior of the NPs.

In this study, flow cytometry profiling of epidermal growth factor receptor (EGFR) expression is demonstrated using gold nanorods that are functionalized with an EGFR-specific antibody. Scattering by this label is primarily observed at 633 nm, within the peak resonance band of the nanorods. A reduced level of nanorod scattering is also seen at 488 nm, below the wavelength range of peak resonance. Additional experiments are also presented to confirm molecular specificity of the new marker.

7.2 Comparison of plasmonic technique

7.2.1 Methods

Nanorod Preparation

Nanorods were fabricated and conjugated to anti-EGFR and mouse IgG$_1$ as described previously in Section 6.1.1.1. The UV-VIS spectra of the immunolabeled nanorod suspensions (ND1000 Spectrophotometer, NanoDrop, Wilmington, DE) indicated a peak absorbance at 643 nm for the anti-EGFR nanorod conjugates and a peak absorbance at 644 nm for the IgG$_1$ nanorod conjugates. Absorbance intensities were used to estimate the concentration of the nanorod suspensions relative to the concentration of the parent suspension, resulting in a nanorod concentration of $8.12 \times 10^{-11}$.
M for the anti-EGFR conjugates and a nanorod concentration of \(8.27 \times 10^{-11}\) M for the IgG\(_1\) conjugates.

**Cell Lines**

Experiments were performed that used flow cytometry to compare receptor expression between antibodies that were either conjugated with gold nanorods or fluorescent labels. The cell lines used for flow cytometry were: (1) MDA-MB-468 breast adenocarcinoma cells that overexpress the EGF receptor; and (2) MDA-MB-453 breast carcinoma cells that do not express the EGF receptor and acted as a negative control. MDA-MB-468 cells were incubated at 37°C and 5% CO\(_2\) using MEM Alpha Medium, supplemented with 30 mL fetal bovine serum and 1% penicillin streptomycin. MDA-MB-453 cells were incubated at 37°C using Leibovitz L-15 Medium, with 50 mL fetal bovine serum and 1% penicillin streptomycin. Both cell lines were obtained from the ATCC through the Duke Cell Culture Facility.

**Flow Cytometry**

Both live cell and fixed cell suspensions were used for flow cytometry experiments using both labeling techniques. Cells were labeled with nanorods by incubating \(5 \times 10^5\) cells with 0.5 mL antibody-conjugated nanorods and 0.5 mL of PBS containing 5% FBS (PBS/FBS) for 20 minutes at room temperature while shaking.
Samples designated for fluorescence labeling were prepared by exposing $5 \times 10^5$ cells to a mixture of 8 µL of anti-EGFR Alexa Fluor 488 conjugate (16-246, clone LA1, Millipore, Billerica, MA) and 1 mL of PBS/FBS for 20 minutes at room temperature under shaking. For control experiments IgG1 Alexa Fluor 488 conjugate (16-240, Millipore, Billerica, MA) was used. Following incubation with a fluorescently labeled antibody, the conjugated cells were washed twice with PBS/FBS solution. For live cell experiments, cells were then resuspended in 0.5 mL PBS/FBS solution. For fixed cell experiments, cells were resuspended in 0.5 mL 1% formalin. Samples were placed in a refrigerator at 4°C and cytometry experiments were conducted within a three hour window.

Titration experiments were performed to determine optimal concentrations for binding saturation. Eight serial dilutions (50%) were prepared for both the anti-EGFR Alexa Fluor 488 and anti-EGFR nanorod conjugates. Each dilution was then profiled with the flow cytometer to measure relative fluorescence or nanorod scattering intensity. Experiments determined that binding saturation occurs with the implemented 8 µL of anti-EGFR Alexa Fluor 488, but not with the available concentrations of anti-EGFR nanorods (Figure 7.1).
Flow cytometry was performed on a MoFlo XDP Cell Sorter (Beckman Coulter) that is capable of detecting 100,000 events per second. Forward and side scattering illumination was provided by a 488 nm solid state laser light source operating at 200 mW. The source dedicated to NP illumination was a 632.8 nm He-Ne laser operating at 25 mW. A 632.8 nm source was selected for excitation because it spectrally aligns within the peak resonance band of the nanorods, providing increased scattering intensity and contrast. The NP scattering was collected and isolated using a neutral density (ND) 2.0 635/10 nm APC filter, while the Alexa Fluor 488 emission (519 nm) was collected and isolated using a 530/40 nm filter. Data was analyzed using FlowJo 9.0.2 software.
7.2.2 Results

The receptor expression in live cells was evaluated and compared between antibody conjugated nanorod labels and Alexa Fluor 488 fluorescent labels using flow cytometry. The fluorescence detector of the flow cytometer acquired both immunolabeled NP side scatter and fluorophore emission. For NP labeled cells, the 635/10 nm filter detected the largest change in scattering between experimental and control groups (Figure 7.2A). EGFR-expressing (EGFR(+)) MDA-MB-468 cells exposed to anti-EGFR nanorods had a mean scattering intensity of 1806 (CV = 29.5, N=10419) (Table 1). MDA-MB-468 control cells exposed to IgG₁ nanorods had a mean scattering intensity of 290 (CV = 26.1, N=11021). The increase represents a 6.23-fold enhancement in scattering by cells labeled with anti-EGFR nanorods over anti- IgG₁ labeled control cells. Using Overton cumulative histogram subtraction of the 635 channel, the percentage of positive cells was determined to be 98.20% for EGFR-expressing (EGFR(+)) MDA-MB-468 cells [148]. According to the Probability Binning (PB) comparison algorithm, the two cell population distributions were different with 99% confidence (p < 0.01, T(χ) = 2812), demonstrating statistical significance [149, 150]. The cell population distributions as observed by the 530 channel were indistinguishable (p = 0.5, T(χ) = 0), confirming that use of a spectral channel matched to the nanorod excitation peak was essential in observing the labeling.
Figure 7.2: Comparison of EGFR binding by gold nanorod (A) and Alexa Fluor 488 (B) labeled antibodies. Anti-EGFR conjugated with nanorods or Alexa Fluor 488 that are bound to EGFR-positive MDA-MB-468 cells are labeled EGFR+/EGFR+ in the figure. Controls consisted of MDA-MB-468 cells bound to IgG$_1$ conjugated nanorods / Alexa Fluor 488 (EGFR+/IGG+), EGFR(-) MDA-MB-453 cells bound to anti-EGFR conjugated nanorods / Alexa Fluor 488 (EGFR-/EGFR+), and unbound MDA-MB-468 cells (EGFR+/0).

Table 7.1: Scattering intensity of cells bound to anti-EGFR gold nanorods and appropriate controls

<table>
<thead>
<tr>
<th>Nanorod Labeled Cells</th>
<th>Mean</th>
<th>CV</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-468 (EGFR+) / anti-EGFR</td>
<td>1806</td>
<td>29.5</td>
<td>10419</td>
</tr>
<tr>
<td>MDA-MB-468 (EGFR+) / anti- IgG$_1$</td>
<td>290</td>
<td>26.1</td>
<td>11021</td>
</tr>
<tr>
<td>MDA-MB-468 (EGFR+) / None</td>
<td>276</td>
<td>23.2</td>
<td>10737</td>
</tr>
<tr>
<td>MDA-MB-453 (EGFR-) / anti-EGFR</td>
<td>244</td>
<td>22.8</td>
<td>15204</td>
</tr>
<tr>
<td>MDA-MB-453 (EGFR-) / anti- IgG$_1$</td>
<td>250</td>
<td>27.8</td>
<td>15179</td>
</tr>
</tbody>
</table>

Table 7.2: Fluorescent intensity of cells bound to anti-EGFR Alexa Fluor 488 and appropriate controls

<table>
<thead>
<tr>
<th>Alexa Fluor 488 Labeled Cells</th>
<th>Mean</th>
<th>CV</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-468 (EGFR+) / anti-EGFR</td>
<td>547</td>
<td>57.3</td>
<td>10645</td>
</tr>
<tr>
<td>MDA-MB-468 (EGFR+) / anti- IgG$_1$</td>
<td>6.67</td>
<td>78.7</td>
<td>16133</td>
</tr>
<tr>
<td>MDA-MB-468 (EGFR+) / None</td>
<td>6.53</td>
<td>72.2</td>
<td>10737</td>
</tr>
<tr>
<td>MDA-MB-453 (EGFR-) / anti-EGFR</td>
<td>7.1</td>
<td>193</td>
<td>15343</td>
</tr>
<tr>
<td>MDA-MB-453 (EGFR-) / anti- IgG$_1$</td>
<td>6.26</td>
<td>1192</td>
<td>19730</td>
</tr>
</tbody>
</table>
For Alexa Fluor 488 labeled cells, the 530/40 nm fluorescent filter detected the largest change in emission between experimental and controls groups (Figure 7.2B). EGFR(+) MDA-MB-468 cells exposed to anti-EGFR Alexa Fluor 488 had a mean fluorescence intensity of 547 (CV = 57.3, N=10645) (Table 2). MDA-MB-468 control cells exposed to IgG1 Alexa Fluor 488 had a mean fluorescence intensity of 6.67 (CV = 78.7, N=16133). The increase represents a 82.0-fold enhancement in emission over IgG1 control cells. Applying Overton subtraction to the 530 channel, the percentage of positive cells was determined to be 98.92% for EGFR-expressing (EGFR(+)) MDA-MB-468 cells. The two cell population distributions were different with 99% confidence (p < 0.01, T(χ) = 2873), demonstrating statistical significance according to the PB comparison. The cell population distributions as observed by the 635 channel were also different with 99% confidence (p < 0.01, T(χ) = 316).

While cellular and nanorod scattering of 632.8 nm incident light was monitored using the fluorescence detector, scattering of 488 nm incident light was detected using traditional forward and side scattering detectors. A slight increase was observed in 488 nm side scatter from anti-EGFR nanorod bound cells compared to IgG1 controls. EGFR-expressing (EGFR(+)) MDA-MB-468 cells exposed to anti-EGFR nanorods had a mean side scatter intensity of 1377 (CV = 17.6, N=10419), while those exposed to IgG1 nanorods had a mean side scatter intensity of 1229 (CV = 21.4, N=11021) (Figure 7.3). This 12% increase in side scatter was not consistently present in all trials and does not obstruct
live cell and doublet population discrimination. Titration experiments demonstrated that the increase in side scatter due to the nanorod tags decreased with reduction of nanorod concentration. The observed change in side scattering at 488 nm reflects the spectral properties of these particular nanorod labels, which exhibit a minor scattering peak near 500 nm. There was no observed trend in forward scatter associated with nanorod concentration.

![Figure 7.3: Comparison of forward and side scatter profiles at 488 nm between MDA-MB-468 cells (EGFR+) labeled with anti-EGFR (EGFR+) and IgG\textsubscript{1} (IGG1+) conjugated nanorods. (Anti-EGFR label: FS Mean=2096, CV = 24.9; SS Mean=1377, CV=17.6. Anti- IgG\textsubscript{1} label: FS Mean=2286, CV=23.9; SS Mean=1229, CV=21.4.)](image)

Experiments were also performed to directly image cell uptake by the conjugated nanorods using darkfield microscopy. Multiple darkfield spectra were acquired from each of 25 randomly selected EGFR(+) MDA-MB-468 cells exposed to anti-EGFR conjugated nanorods. Observed cells exhibited significant conjugate binding (Figure...
7.4A and Figure 7.5). Collected spectra had a peak mean scattering intensity of 0.16 ± 0.10 (A.U.) (N=117) centered at 668.7 ± 9.8 nm (N=109) with a mean FWHM of 140.3 nm (N=109) (Figure 7.5).

Figure 7.4: Darkfield images of A) MDA-MB-468 cells exposed to anti-EGFR conjugated nanorods, B) Fluorescence microscopy image of MDA-MB-468 cell labeled with anti-EGFR Alexa Fluor 488 and C) DIC image of unlabeled MDA-MB-468 cell. (Scale bars = 20 µm)

Figure 7.5: Spectral scattering profiles of MDA-MB-468 cells (EGFR+) exposed to anti-EGFR conjugated nanorods.
7.3 Discussion of advantages of nanorod approach

Nanorods with dimensions of 67.1 ± 8.9 nm in length and 32.0 ± 6.1 nm in diameter (N=114) were chosen for this study for several reasons. First, membrane-bound nanorods of this size exhibit a scattering peak centered at 668.7 ± 9.8 nm (N=109), a wavelength that is greater than the expected range of endogenous scattering from cells and their constituents [16]. It is noted that the bimodal resonance of nanorods also provides a smaller, blue-shifted secondary peak which can be exploited for detection at an alternative wavelength. Second, these nanorods are not expected to significantly alter the general shape of the cell [147]. Third, there is reduced scattering of 488 nm incident light for a nanorod of this size, minimizing changes in forward and side scattering at this wavelength, such that this labeling will not significantly interfere with complementary measurements of cell size and granularity by flow cytometry. Fourth, the gold nanorods chosen for this study have very large scattering cross sections at their plasmon resonance wavelength, making them ideal tags compared to other NPs used in previous studies which were not matched to the excitation wavelength [143, 145]. Finally, gold nanorod markers can be fabricated to scatter across a broad spectral range, up to 2000 nm, unlike gold nanosphere markers which are limited to visible spectra.

The results presented herein show that immunolabeled nanorods can be used to measure relative receptor expression using flow cytometry with a discrimination of expressing populations comparable to fluorescently labeled antibodies while offering
some potential advantages. The anti-EGFR nanorod labeled EGFR-positive cells exhibited a 6.23-fold increase in scattering over cells labeled with the anti IgG₁ nanorods, demonstrating the applicability of this technique for analysis of cell populations. Commercially available Alexa Fluor 488 conjugate demonstrated a larger, 82.0-fold increase in emission over the IgG₁ control group. Despite this disparity in separation, the anti-EGFR nanorod label still identified an expressing population of 98.20%, relative to the anti- IgG₁ nanorod control, while the anti-EGFR Alexa Fluor 488 label identified an expressing population of 98.92% relative to the anti- IgG₁ Alexa Fluor 488 control. In addition, the anti-EGFR nanorod label demonstrated a higher precision with a CV = 29.5, relative to the anti-EGFR Alexa Fluor 488 with a CV = 57.3.

The lower increase in signal observed for nanorod labeled cells (6.23) versus fluorescently labeled cells (82.0) is predominantly attributed to two factors. First, fluorescent tags are implemented in flow cytometry with system filters that remove the incident absorption wavelength and isolate the shifted emission wavelength, as was the case with the anti-EGFR Alexa Fluor 488 measurements here. When using NPs as labels, scatter at the incident wavelength produces a non-zero baseline, resulting in an artificially high scattering observed for unlabeled control cells. This baseline has the effect of reducing the relative increase in scattering observed for labeled experimental cells. Applications such as darkfield microscopy avoid this limitation by removing incident light optically, using an iris for example. This is accomplished in flow
cytometry using an obscuration bar. However, this mechanism only partially removes
the illumination, which results in higher negative control peak intensities, despite low
photomultiplier tube (PMT) and high ND filter settings. Therefore, the anti-EGFR
nanorod increase observed here may actually underestimate the performance of the
label. Subtraction of the baseline mean scattering intensity (276) seen for unlabeled
(EGFR(+)) MDA-MB-468 cells results in a mean intensity of 1530 for the anti-EGFR
nanorods, and 14 for IgG1 labeled nanorods. Accounting for this unfiltered illumination
results in a much larger 122.63-fold increase, demonstrating a sensitivity for the nanorod
label comparable to the fluorescent label.

A second factor which must be considered in comparing the two labeling
methods is that the concentration of nanorod conjugated antibody was one thousand
fold lower than the fluorescently labeled antibody. The nanorod concentration of the
anti-EGFR conjugates was $8.12 \times 10^{-11}$ M while that of Alexa Fluor 488 conjugate had an
antibody concentration of $2.89 \times 10^{-8}$ M and a fluorescent molecule to antibody ratio of 4:1
(mol:mol), resulting in a fluorophore concentration of $1.16 \times 10^{-7}$ M. The relatively lower
concentration of nanorods is due to the limitations of the nanorod fabrication protocol.
As a result, binding of the nanorods did not reach saturation, unlike the fluorescent
labels. Despite this large disparity in concentration and saturation level, significant
scattering intensities were still observed in flow cytometry using the nanorods and a
relatively high contrast was observed in darkfield microscopy for EGFR-positive cells incubated with the anti-EGFR labeled nanorods.

In these experiments, the Alexa Fluor 488 labels are conjugated to clone LA1 of anti-EGFR while the nanorods are conjugated to clone 225. Clone LA1 recognizes an antigenic determinant on the extracellular domain of the receptor. It is unknown which specific epitope clone 225 recognizes. The potential differences in avidities associated with these two implemented anti-EGFR clones may have also been a factor which contributed to the lower performance of the nanorod conjugates. However, it is more likely the lack of saturation conditions would remain as the limiting factor to nanorod conjugate binding.

If the increased background illumination is not properly accounted for, the signal enhancement obtained with the nanorod labeling technique can be seen as slightly reduced relative to previous NP cytometry studies which achieved ten-fold increases [143, 151], but can still offer some advantages. For instance, in an initial study that used immunolabeled 40 nm gold nanospheres to observe scattering of 632.8 nm incident light, a ten-fold enhancement was observed between experimental and control groups [143]. Mie theory modeling indicates that such an enhancement is not possible without plasmonic coupling between the NPs. To achieve plasmonic coupling, cells would need to be exposed to a large concentration of NPs, ensuring conjugate binding of the majority of surface membrane receptors. In our samples, scattering spectra matched
theoretical values predicted by DDA modeling for single, isolated nanorods, indicating negligible plasmonic coupling [23, 24]. This may be a confounding factor since the use of a large number of NP labels can have an adverse effect on cell shape, potentially skewing the traditional flow cytometry measurements of forward and side scatter. Our approach enables this effect to be monitored by employing both 488 nm and 632.8 nm sources, unlike this previous study. The nanorod conjugate scatters strongly under 632.8 nm incident light, but has minimal scattering at 488 nm. The reduced scattering of the nanorods at 488 nm preserves the ability of the cytometry system to evaluate relative cell granularity and intracellular structures, while the increased 632.8 nm scattering provides a method of differentiation of receptor expression.

Another advantage of using NPs in flow cytometry applications is the potential for achieving sensitivity comparable to fluorophores. Current commercial flow cytometers are capable of detecting a minimum level of a few hundred fluorescein molecules per particle. Some custom cytometry systems have reported single fluorophore molecule detection, but this level of sensitivity is not widely available [152]. With single NP scattering intensities on the order of $10^5$ fluorescein molecules, commercial flow cytometers theoretically can detect single NP labels [153]. By enabling detection at the limit of a single NP, the detection of a single labeled cell-surface receptor by flow cytometry could be realized. Additional experiments are required to quantitatively compare sensitivity between the two label types in a practical application.
The application of NPs in flow cytometry provides simple avenues for further signal enhancement. The scattering intensity of NPs can be amplified through a variety of approaches. The simplest method is to conjugate the antibody of interest to a NP which exhibits a larger relative scattering cross section at the source wavelength. A second approach is to increase the concentration of NPs implemented, allowing for binding saturation to occur. As the number of bound NPs increases there is initially a linear increase in scattering intensity [73]. At higher concentrations, however, the distances between membrane-bound NPs decreases causing plasmonic coupling and a subsequent quadratic increase in scattering intensity [17]. On average, EGFR expressing cells contain between $2 \cdot 10^4$ to $2 \cdot 10^5$ EGFR proteins per cell [154]. This number of potential binding sites offers a significant source for further enhancing the NP scattering signal. Finally, in multi-parametric applications where both NPs and fluorophores are applied, NPs can be used to enhance fluorescence intensity by up to fifty-fold by choosing a NP with a peak resonant wavelength corresponding to the fluorophore emission [155].

### 7.3.1 Application of supercontinuum source in flow cytometry

Another means for enhancing the utility of nanorods for flow cytometry is to employ broadband, high power light sources such as supercontinuum sources. A recent study demonstrated the use of a single broadband super continuum source with high spectral density for flow cytometry with fluorescent labels [156]. Such sources would
allow for the use of multiple specific excitation wavelengths (420 nm - 2000 nm), greatly expanding the number of contrast agents available, while drastically reducing the number of line sources, cytometer size, and cost. The high spectral density of 1-3 mW/nm translates to fluorescent excitation power levels of 15-40 mW, comparable to current flow cytometer individual laser sources [156]. Additionally, these alternative sources do not suffer from the limitations of arc lamps and LEDs, including low power and required complex optical trains. If these sources gain acceptance moving forward, there will be 1400 nm of bandwidth readily available for nanorods which have already been developed. These nanorods do not pose cytotoxicity concerns as one would expect with quantum dots, the alternative near-infrared label. While other issues may arise in this spectral region, including noisy IR detectors and the presence of water absorption, these could be addressed with further technological advances. In our experiments, the variation of RI on the cell membrane resulted in a broadening of spectral width (FWHM) of the nanorod labels from approximately 80 nm in suspension, to 140 nm upon binding. Nonetheless, this still presents the possibility to allow 10 multiplexed nanorod labels to scatter within the spectra provided by this new generation of sources.

In conclusion, plasmonic nanorods show great promise for application to flow cytometry. The unique spectral properties of plasmonic NPs provide advantages including spectral agility, biocompatibility and non-quenching optical properties. In addition, nanorod scattering, extending well into the infrared spectrum, significantly
increases the spectral range available for cytometry, which had previously been largely limited to visible wavelengths when using solely fluorescent labels or gold nanospheres. The ability to assess receptor expression through flow cytometry is demonstrated using immunolabeled nanorods with a sensitivity and precision comparable to fluorescent labels. Statistically significant differences in scattering intensity were observed, which allowed differentiation of receptor positive and receptor-negative cell populations. The minimal scattering of the nanorods at 488 nm also maintained the ability to characterize relative cellular size and granularity using traditional flow cytometry scatter measurements.
8 Conclusions and Future Directions

The projects described herein involved the development of molecular imaging systems, NP biomarkers, optical sensing techniques, and scattering models with direct implications and application towards a multitude of future projects. While the scope of the reported studies was narrow in some cases, each work provided additional insight and tools toward future research in this area.

Foremost, three spectrally distinct NPs tags of various shape, size, and composition were developed and demonstrated for molecular imaging and optical sensing applications. Each label was functionalized with an antibody towards a receptor commonly overexpressed in metastatic cancer. The conjugates were then characterized and implemented in vitro to demonstrate molecular specificity. These advances alone provide the tools necessary for various applications.

In these experiments, the developed labels were primarily implemented towards in vitro optical sensing and molecular imaging applications. While previous dielectric sensing studies were limited to anti-EGFR coated gold nanospheres, the experiments outlined here demonstrated the use of anti-IGF-1R silver nanospheres and anti-EGFR gold nanorods towards dielectric sensing for the first time, providing local cellular RI values typical of the cellular membrane environment. HER-2 Ab gold nanospheres additionally provided insight toward potential avenues for dimer monitoring. More
importantly, because the particles have significant differences in peak scattering, they are suitable for simultaneous implementation in multiplex applications. While experiments demonstrated different permutations for immunophenotype profiling with molecular imaging experiments, there are extending applications that would further benefit from this work.

One such application is photothermal therapy, which employs lasers towards the treatment of tumors. The technique is based around delivering or generating sufficient levels of heat to cancerous tissue, while simultaneously avoiding damage of healthy areas. The increased temperature leads to hyperthermic conditions resulting in denaturing of proteins, loosening of cell membranes, and eventual cell death. Functionalized noble metal NPs with large absorbance cross sections allow for localized heating using small amounts of delivered energy, reducing damage to nearby healthy tissue while easily raising cancerous tissue past destructive threshold temperatures [157]. Targeting with multiple NPs provides the technique with two additional advantages. First, when targeting metastatic tissue phenotypes that overexpress all three receptors, there will be a significant increase in specificity. Second, with distinct resonance peaks, more energy can be delivered at multiple absorbance wavelengths. Furthermore, in Section 6.3 the red-shifted peak resonance of a NP pair undergoing plasmonic coupling is characterized. The shifted resonance could allow for the targeting of cells that are exhibiting phenotypes with increased dimer formation. Such targeting is
not necessarily limited to cancer therapy applications. Selective damage of receptors exhibiting a particular behavior may be suitable for the recreation of conditions of a particular disease.

As mentioned in Section 2.4.2, “lab in a tube” biosensor have been explored recently [35]. These assays consist of a colloid suspension containing three distinct NPs functionalized to different antigens. Upon ligand binding, an observable shift is exhibited on the respective NP spectra. Previous works is somewhat limited, but some of the results address the issues encountered in our work. For instance, such a setup will not suffer from a change in particle concentration upon receptor binding. Therefore, if conjugates are chosen appropriately, low receptor expression will not result in masking by the over-expressed receptor. The labels and cell lines studied here can easily be applied towards this technique, simply by collecting and preparing the necessary membrane protein.

The receptor quantification and immunophenotype studies suffered from a lack of receptor expression distribution information. Flow cytometry represents the standard method for distribution profiling. Experiments were conducted to demonstrate profile analysis using anti-EGFR gold nanorods. The next step would be to implement the previously developed NPs towards a multiplexing experiment using flow cytometry. Each NP tag has already been matched to source wavelengths readily available with current flow cytometers. Additionally, recent work has shown the use of broadband
supercontinuum sources that extend the spectrum and availability for multiple tags in the near-infrared region. Nanorods exhibit peak resonances covering this extended range, providing enhanced multiplexing ability. Much work was spent both developing the correct nanorod peak resonances and implementing a super continuum source in our setup. The combination of both of these in a flow cytometer system would demonstrate the further multiplexing capability provided by these technologies.

While developing the functionalized silver nanosphere label, interesting behavior was observed. The particles themselves had very large scattering cross sections resulting in high contrast. The high contrast occurs despite an apparent lower binding efficiency, with the total number of bound particles remaining low, as seen in the images in Chapter 6. Such characteristics are perfect for single particle tracking applications where significant binding of the colloid is undesired. Time-resolved single particle tracking would allow for the monitoring of peak resonance changes as the receptor is uptaken through endocytic vesicles.

Of course, monitoring of such dynamic processes would require a hyperspectral imaging system capable of high speed acquisition to spectrally track the particle as it undergoes receptor-mediated endocytosis. The imaging system developed in Chapter 3, while sufficient for the applications described in Chapter 6, would require further development to take advantage of the high CCD acquisition speeds. If the system were
to be used in its current configuration, a multi-spectral approach might be more suitable, imaging at specific wavelengths of interest rather than an extended spectral range.

Significant effort was spent exploring polarization mapping of proximal NPs as a tool to discern plasmonic coupling effects from dielectric sensing in Chapter 5. Experiments indicate the ability to separate the two orthogonal scattering modes, but theory clearly shows plasmonic coupling effects still occur in the transverse mode. Therefore, the advantage of the technique lies in the theoretically demonstrated enhanced dielectric sensing application, particularly in cases where there is significant overlap between the two mode peaks, which occurs when there is mid-range separation between the proximal particles. The HER-2 gold nanosphere experiments of Section 6.3 revealed interparticle distances falling within this range. An interesting follow-on study could isolate the longitudinal scattering modes from these particles using the polarization mapping technique to obtain enhanced sensing. Alternatively, smaller nanosphere conjugates could be implemented that do not necessarily touch during dimerization, allowing for measurement of interparticle spacing and comparison with standard techniques such as FRET.

In conclusion, various applications benefit from the novel tools and techniques presented herein. While demonstrated experiments were mostly limited to in vitro molecular imaging and RI sensing, the findings have implications in a broader range of areas. These applications include, but are not limited to photothermal therapy, biosensor
assays, flow cytometry, particle tracking, enhanced dielectric sensing, and monitoring of intermolecular sensing.
9 References


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