AN EXPLORATION OF CELL RECEPTOR LABELING VIA DARK FIELD IMAGING AND QUANTIFYING DENSELY BOUND SERS LABELS VIA RAMAN SIGNAL STRENGTH

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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An exploration of cell receptor labeling via dark field imaging
and quantifying densely bound SERS labels via Raman signal strength

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Abstract

Two experiments explore the application of plasmonic nanoparticles to cellular pathology. The first devised a platform by which gold-silver nanoparticles act as differentiable labels for cell surface receptors under dark field imaging. By conjugating particles of various constitutions with receptor-targeting antibodies, particles scatter characteristically according to their plasmon peak. The second experiment programmed receptor placement via the patterning of two substrates and used the binding of SERS nanoparticles to explore the quantification of such targets at high-density. On one substrate, anchor pairs established receptors at specified distances in order to define the relationship between scattering intensity and the distance between SERS particles. On the second, anchor regions are filled with increasing densities of receptors and the particle-saturated substrates are probed to relate scattering intensity to particle density. This should discover the density-threshold between linear and non-linear scattering and inform the quantification of particles in the exponential density regime.
Acknowledgements

It is useless to attempt to reason a man out of a thing he was never reasoned into.
- Jonathan Swift

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# Table of Contents

**Acknowledgements** ........................................................................................................ iii

**Table of Contents** ........................................................................................................ iv

**Table of Tables** ............................................................................................................ vii

**Table of Figures** .......................................................................................................... viii

**Table of Equations** ...................................................................................................... x

**Introduction** .................................................................................................................. 1

  **Motivation** .................................................................................................................... 1

  **Overview of experiments** ............................................................................................ 1

**Cell surface receptor dark field labeling via plasmonic alloy nanoparticles** .................. 3

  **Motivation and Technological overview** .................................................................... 3

  **Existing work** ............................................................................................................. 3

**Dark field microscopy** ................................................................................................. 4

  **Dark field condenser (as used)** .................................................................................. 4

  **via proximate fibre with a focused output** ................................................................. 5

**Scattering Theory** ....................................................................................................... 6

  **Plasmonic nanoparticles** ............................................................................................ 7

**Gold/Silver alloy nanoparticles (and their synthesis)** ..................................................... 8

  **Synthesis** ................................................................................................................... 8

**Immunological targeting** .............................................................................................. 10

**Nanoparticle functionalization** .................................................................................... 10

  **Thiol bonding** ............................................................................................................ 11

  **EDC/NHS Coupling** ................................................................................................ 11

  **Sample preparation for imaging** ............................................................................... 13

  **On: Slide Cleaning** ................................................................................................... 13

  **Bulk defects** ............................................................................................................. 14

  **Surface Roughness** ................................................................................................... 15

  **Cell handling** .......................................................................................................... 15

  **Spectral analysis** ....................................................................................................... 16

**Equipment:** ................................................................................................................. 17

  **Optical microscope** .................................................................................................. 17
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Functionalization/Sample preparation</td>
<td>17</td>
</tr>
<tr>
<td>Solutions used:</td>
<td>18</td>
</tr>
<tr>
<td>PEGylate nanoparticles</td>
<td>18</td>
</tr>
<tr>
<td>EDC/NHS activation</td>
<td>18</td>
</tr>
<tr>
<td>Conjugate antibodies to nanoparticles</td>
<td>19</td>
</tr>
<tr>
<td>Incubate NPs with cells</td>
<td>19</td>
</tr>
<tr>
<td>Mounting sample for microscopy</td>
<td>19</td>
</tr>
<tr>
<td>Experimental results</td>
<td>19</td>
</tr>
<tr>
<td>Evidence of targeting (60 nm Au NPs): Imaging</td>
<td>19</td>
</tr>
<tr>
<td>Evidence of targeting (60 nm Au NPs): Flow cytometry</td>
<td>21</td>
</tr>
<tr>
<td>Evidence of differentiability (AgAu alloy NPs)</td>
<td>23</td>
</tr>
<tr>
<td>Evidence of targeting (AgAu alloy NPs)</td>
<td>25</td>
</tr>
<tr>
<td>Discussion:</td>
<td>27</td>
</tr>
<tr>
<td>The differentiability of alloy nanoparticles according to their constitution</td>
<td>27</td>
</tr>
<tr>
<td>That alloy nanoparticles can be coimaged alongside cells</td>
<td>28</td>
</tr>
<tr>
<td>That immunotargeting by nanoparticles is possible [while retaining signal integrity]</td>
<td>28</td>
</tr>
<tr>
<td>Quantifying densely-bound SERS labels</td>
<td>30</td>
</tr>
<tr>
<td>Motivation and Strategy:</td>
<td>30</td>
</tr>
<tr>
<td>Past work</td>
<td>31</td>
</tr>
<tr>
<td>Substrate patterning</td>
<td>31</td>
</tr>
<tr>
<td>“FormatFlow”: From software to software to hardware</td>
<td>31</td>
</tr>
<tr>
<td>Dynamic functions: Stencils and Pairs</td>
<td>32</td>
</tr>
<tr>
<td>On: Patterning</td>
<td>32</td>
</tr>
<tr>
<td>The Surface</td>
<td>33</td>
</tr>
<tr>
<td>Fabrication</td>
<td>33</td>
</tr>
<tr>
<td>Functional</td>
<td>33</td>
</tr>
<tr>
<td>!Coupling</td>
<td>34</td>
</tr>
<tr>
<td>Functionalization procedure</td>
<td>34</td>
</tr>
<tr>
<td>Targeting System</td>
<td>34</td>
</tr>
<tr>
<td>SERS label functionalization</td>
<td>34</td>
</tr>
<tr>
<td>Particle Imaging</td>
<td>35</td>
</tr>
<tr>
<td>Confocal raman microscopy</td>
<td>36</td>
</tr>
</tbody>
</table>
**Table of Tables**

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1 - Fabrication procedure for the anchor site pair platform</td>
<td>40</td>
</tr>
<tr>
<td>Table 2 - Fabrication procedure of the variable density platform</td>
<td>52</td>
</tr>
</tbody>
</table>
# Table of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Diagram of a typical dark field setup&lt;sup&gt;31&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Diagram of dark field setup using a directed proximate source</td>
</tr>
<tr>
<td>3</td>
<td>Extinction spectra for various constitutions of AgAu alloy NPs&lt;sup&gt;34&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>XPS elemental analysis of alloy NPs&lt;sup&gt;34&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>Overall functionalization procedure for plasmonic NPs</td>
</tr>
<tr>
<td>6</td>
<td>Reaction pathways of free -COOH with EDC/Sulfo-NHS. Reprinted (adapted)</td>
</tr>
<tr>
<td>7</td>
<td>Functionalization outcomes for the reaction of -COOH with various concentrations of EDC/Sulfo-NHS; outcomes defined by overlapping regions of terminal products. Reprinted (adapted)</td>
</tr>
<tr>
<td>8</td>
<td>Dark field image of patient leukocytes mounted poorly</td>
</tr>
<tr>
<td>9</td>
<td>Dark field image of patient leukocytes handled appropriately and mounted with CFM-1</td>
</tr>
<tr>
<td>10</td>
<td>Dark field image of a dense collection of 60 nm Au NPs</td>
</tr>
<tr>
<td>11</td>
<td>Spectrum corresponding to scattering from dense collection of 60 nm Au NPs (λ&lt;sub&gt;p&lt;/sub&gt;=550 nm)</td>
</tr>
<tr>
<td>12</td>
<td>Dark field image of patient leukocytes incubated with untargeted Au NPs (-)</td>
</tr>
<tr>
<td>13</td>
<td>Dark field image of patient leukocytes incubated with targeted Au-CD45 NPs (+)</td>
</tr>
<tr>
<td>14</td>
<td>Flow cytometry results for fluorescence signal from post-stained untargeted cells (-)</td>
</tr>
<tr>
<td>15</td>
<td>Flow cytometry results for fluorescence signal from post-stained cells saturated with anti-CD45 antibodies (+)</td>
</tr>
<tr>
<td>16</td>
<td>Flow cytometry results for fluorescence signal from post-stained cells saturated with filtered anti-CD45 antibodies (+)</td>
</tr>
<tr>
<td>17</td>
<td>Flow cytometry results for fluorescence signal from post-stained cells targeted by anti-CD45 programmed Au NPs (+)</td>
</tr>
<tr>
<td>18</td>
<td>Dark field image of ablated Ag NPs</td>
</tr>
<tr>
<td>19</td>
<td>Dark field image of ablated Ag&lt;sub&gt;0.5&lt;/sub&gt;Au&lt;sub&gt;0.5&lt;/sub&gt; NPs</td>
</tr>
<tr>
<td>20</td>
<td>Dark field image of ablated Au NPs</td>
</tr>
<tr>
<td>21</td>
<td>Dark field image of patient cells incubated with untargeted Au NPs</td>
</tr>
<tr>
<td>22</td>
<td>Dark field image of patient cells incubated with Au-CD45 NPs</td>
</tr>
<tr>
<td>23</td>
<td>Dark field image of patient cells incubated with Ag&lt;sub&gt;0.5&lt;/sub&gt;Au&lt;sub&gt;0.5&lt;/sub&gt;-CD45 NPs</td>
</tr>
<tr>
<td>24</td>
<td>Dark field image of patient cells incubated with Ag-CD45 NPs</td>
</tr>
<tr>
<td>25</td>
<td>Schematic of lithographic process&lt;sup&gt;64&lt;/sup&gt;</td>
</tr>
<tr>
<td>26</td>
<td>SERS spectra from exciting a sample label at available wavelengths</td>
</tr>
<tr>
<td>27</td>
<td>SEM image (VP mode) of 60nm Au NPs</td>
</tr>
<tr>
<td>28</td>
<td>Wafer-level image of CAD for anchor site pair platform</td>
</tr>
<tr>
<td>29</td>
<td>Chip-level image of CAD for anchor site pair platform</td>
</tr>
<tr>
<td>30</td>
<td>Image of CAD for anchor site pair platform focusing on the corner sub-arrays for four separation distances</td>
</tr>
<tr>
<td>31</td>
<td>Image of CAD for anchor site pair platform focusing on the corner subarray where anchor sites are separated by 55 nm</td>
</tr>
<tr>
<td>32</td>
<td>CAD image for anchor site pair platform focusing on a single pair (55 nm separation)</td>
</tr>
<tr>
<td>33</td>
<td>SEM image of anchor pair from preliminary dose test (CtC: 68.1 nm)</td>
</tr>
<tr>
<td>34</td>
<td>SEM image of anchor pair from preliminary dose test (CtC: 99.1 nm)</td>
</tr>
<tr>
<td>35</td>
<td>SEM image of anchor pair from preliminary dose test (CtC: 128.1 nm)</td>
</tr>
<tr>
<td>36</td>
<td>SEM image of anchor pair from preliminary dose test (CtC: 152.1 nm)</td>
</tr>
<tr>
<td>37</td>
<td>Expected relationship between SERS scattering intensity from a pair of SERS particles and the distance separating the particles to be derived from anchor pair platform&lt;sup&gt;60&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Figure 41 - Wafer-level image of CAD describing variable density platform  
Figure 42 - Chip-level image of CAD describing variable density platform  
Figure 43 - Image of CAD describing variable density platform focusing on an indexed array of anchor sites  
Figure 44 - Photograph of finished photolithography mask for variable-density platform  
Figure 45 - Photograph of finished photolithography mask for variable-density platform focused on indexed anchor sites  
Figure 46 - Photograph of diced wafer for use in variable-density experiments  
Figure 47 – Light microscopy image of diced wafer for use in variable-density experiments focused on intersection between four chips  
Figure 48 - Light microscopy image of chip for use in variable-density experiments focused on indexed anchor sites (20x)  
Figure 49 - Light microscopy image of chip for use in variable-density experiments focused on indexed anchor sites (40x)  
Figure 50 - SERS scattering intensity with increasing density of receptors
### Table of Equations

<table>
<thead>
<tr>
<th>Equation</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equation 1</td>
<td>Scattering intensity for spherical particles as per Rayleigh model</td>
<td>6</td>
</tr>
<tr>
<td>Equation 2</td>
<td>Scattering cross-section for spherical particles as per Rayleigh model</td>
<td>7</td>
</tr>
<tr>
<td>Equation 3</td>
<td>Scattering cross-section of a spherical particle in terms of dielectric functions</td>
<td>7</td>
</tr>
</tbody>
</table>
Introduction

Motivation
For the ≈5600 Canadians diagnosed with leukemia every year, prognosis depends on the stage of diagnosis\(^1\). With chronic lymphocytic leukemia (CLL), for example, a stage 0 patient, where preemptive screening detects the cancer, has a median survival rate of 12.5 years, whereas a stage IV patient, now suffering from swollen organs, anemia, low platelet count, and high white blood cell count, has only a median survival rate of 2 to 4 years\(^2-4\). Early diagnosis remains limited, however; CLL is often only noticed with the appearance of elevated white blood cell populations and then diagnosed via morphological analysis and flow cytometry, a population-based screening of fluorescence-tagged cells. Discovery is thus contingent on the cancer being sufficiently advanced to exceed defined signal thresholds – thresholds that require marker concentrations far in excess of what is present in the earliest stages of cancer. Moreover, the interrogation of labels is limited by bleaching and fluorescence spectra so broad that only three or four are distinguishable at once, an obstacle when diagnosis considers a plurality of markers\(^5\). This thesis will explore experiments that seek to improve this diagnostic process. The first imagines a novel platform that would extend current pathology by supplementing morphological study with receptor labeling under dark field microscopy; the second addresses the specific challenge of quantifying Surface Enhanced Raman Scattering (SERS) labels once bound to cell surfaces at high-density.

Overview of experiments
Two sets of experiments will be discussed exploring the application of metal nanoparticles to cellular labeling. Though both are premised on the contemporary application of plasmonic properties and immunotargeting, they rely on fundamentally different optical phenomena and seek to address very different challenges.

Cell surface receptor dark field labeling via plasmonic alloy nanoparticles
Experiments aimed to facilitate Leukemia diagnosis by extending typical morphological analysis to offer visibly multiplexed cell surface receptor labeling while still utilizing a traditional light microscope. Plasmonic nanoparticles which scatter with characteristic colouration are functionalized so as to target specific cell receptors; said receptors are thus visibly labeled when patient cells, incubated with
immunotargeted particles, are imaged under dark field microscopy. Targeted receptors, visible as differentiable coloured points where scattering particles are bound, overlay cells’ morphological outline.

**Particle-binding density of SERS labels**

Measurements over two binding platforms address the challenge of quantifying SERS-active nanoparticles when bound in high densities. Non-linear interactions between particles, though a boon for signal amplification, conflate particle population with position. By first relating SERS scattering from a pair of particles to the distance between the particles ($I_{\text{SERS}} \propto d_p$) then applying this information to a study of SERS scattering over a large area bound with increasing densities of SERS particles, the goal is to experimentally define the interaction distance between particles and the linear regime of binding density then learn to quantify the number of scatterers within an interacting regime.
Cell surface receptor dark field labeling via plasmonic alloy nanoparticles

Motivation and Technological overview
The first sign of leukemia is most often a highly elevated white blood cell count. Once a suspect leukocyte population is established, clinical pathology relies on two core elements to identify and typify the affliction: cell morphology and cell surface receptors. In order to assess the former, current methodology relies on light microscopy to image stained cells and juxtaposes them with references for healthy and malignant cells. Fluorescence-based flow cytometry is then relied upon to recognize receptors that characterize leukemia. By considering both morphology and indentifying receptors with sufficient specificity, leukemia is not only detected by the sub-type is determined. Where the ease and accuracy with which this diagnosis is made is critical to patient prognosis, fluorescence technology has many flaws, namely a weak and bleaching signal and a low ceiling to multiplexing given broad excitation bandwidth.

The proposed platform relies on immunotargeted gold-silver alloy nanoparticles as cell surface receptor labels distinguishable under dark field microscopy. Plasmonic particles exhibit a scattering peak based on their resonance wavelengths and thus, imaged under a dark field light microscope which only captures scattered light, are differentiable by their colour. By using gold-silver alloy nanoparticles, which exhibit resonance peaks between that of pure silver ($\lambda_s=400$ nm) and pure gold ($\lambda_g=520$ nm) according to their constitution, scattering targets are available to manifest any colour between violet and green. By selecting alloys that are easily differentiable by their scattering colour and programming each alloy-type with a different receptor targeting agent, cell receptors can be labeled by incubating the cell sample with the various targeted particles and imaging the result in dark field microscopy. The platform thus offers not only morphological information, as cell membranes scatter clearly, but overlays multiplexed receptor labeling for as many receptors as there are differentiable colours. This will not only facilitate pathology through inherent coimaging but improve the sensitivity of cancer cell detection with the prospect of bright, unbleachable labels that can be imaged at the single particle level.

Existing work
While the remarkable optical properties of colloidal gold have been recognized since the observations of Zsigmondy, recent innovations in the synthesis and application of nanoparticles have attracted ever more attention to the field of plasmonics. Scattering behaviour, understood generally for a spherical
particle through the Mie solutions to Maxwell’s equations was interpreted more specifically for size ranges much smaller than that of the scattering radiation, giving rise to the Rayleigh regime of elastic scattering. Plasmonic behaviour is now modeled through computational electrodynamics, using numerical analysis techniques to find approximate solutions to time-dependent Maxwell’s equations - most notably, the finite-difference time-domain method. The scattering properties of particles have thus been established both theoretically and experimentally for most any configuration of pure, alloyed, or core-shell particle, and dark field light microscopy has been increasingly recognized as a convenient imaging technique to leverage these anomalous scattering behaviours. As efforts to stabilize and functionalize plasmonic particles have succeeded, promising applications have surfaced for their use as biological labels – namely for tissue samples and, more recently, individual cells.

Dark field microscopy

Dark field light microscopy is a relatively simple adaptation of the traditional light microscope that allows it to image by scattered light as opposed to the transmitted light utilized in a conventional bright field setup. Light is focused on the sample at a high angle such that any part of the field that is not obscured by the sample allows light to pass unmolested, appearing as "dark" in the image; in any part of the field of view that is occupied by sample, the sample scatters illuminating light which is then collected in the objective, as per usual. For single layer samples that scatter well, this allows for high contrast images without a complex staining or sample preparation - especially useful with biological samples that offer little inherent contrast in terms of transmission - while still relying on an inexpensive and ubiquitous platform.

The key characteristic of dark field microscopy is its method of illumination: injecting light onto the sample at a high enough angle such that only scattered light will enter the objective [and any transmitted light will pass uncollected by the objective]. This can be accomplished in any number of arrangements depending on the light source, substrate, and optics available, but the angle of incidence of illumination must always exceed the angle of collection of the objective (relative to the normal of the substrate).

Dark field condenser (as used)

This is by far the most common implementation because of its reliance on the bright field microscope setup as a base, requiring only a specialized condenser to provide "high-angle illumination". Instead of a
standard condenser producing a cone of light focused on the sample plane, a circular stop is centered below the condensing optics, blocking all but the outer circumference of the cone - producing a hollow cone of light. The thickness of this cone, shaped by an aperture further down the optical pathway, represents the fundamental trade-off in dark field illumination; it defines both the amount of light that illuminates the sample (surface area of illuminating beam) and the breadth of the angle of illumination. Whereas more light improves imaging (stronger signal), a thinner angle of illumination facilitates achieving a geometry whereby only scattered light is collected (less noise). In optical terms, this need translates to a requirement that the numerical aperture of the condenser exceed that of the objective, though non-idealities - such as the finite thickness of the illuminating cone - will always push this criterion further. Low illumination intensity is a common struggle with this platform as the grand majority of all light must be rejected in order to "hollow" the cone of light emerging from the condenser.

![Diagram of a typical dark field setup](image)

**Figure 1 - Diagram of a typical dark field setup**

via proximate fibre with a focused output

The minimalist option, the light source is coupled with a fibre optic cable terminated with focusing optics. This focused beam is then applied to the sample, typically at very short range and high angle. The light source can thus be positioned on the same side (z-direction) of the sample as the objective, allowing for opaque substrates such as Si; moreover, all the coupled light can be projected from as tight an angle (radially) as the optics allow. This offers a chance for polarized light to be used to probe the orientation of anisotropic samples without the need to reject the "unwanted angles" of illumination at the source. On the other hand, coupling optics necessarily result in a significant loss of intensity and,
without the precise illumination geometry of a standard microscope, an alternative structure is needed to provide stable positioning and minute adjustability.

Figure 2 - Diagram of dark field setup using a directed proximate source

Scattering Theory

The scattering of electromagnetic radiation, solved analytically in terms of waves interacting with a spherical body, is described by solutions to Maxwell's equations known as Mie Theory. For particles, the operative parameter is defined by the particle's size relative to the wavelength of the incoming light, $x = \frac{2\pi r}{A}$. Where $x \ll 1$, Mie Theory collapses to the Rayleigh model, a special case for particles much smaller than the wavelength of the scattering light where interactions are dominated by considerations arising from the electric polarizability of the particle. Incoming electromagnetic radiation polarizes the surface electrons of the scatterer, forcing them to oscillate with a frequency identical to that of the light; the particle thus behaves as per a radiating dipole, scattering light elastically.

Scattering intensity and scattering cross-section are described as follows:

$$I = I_0 \frac{1 + \cos^2 \theta}{2R^2} \left( \frac{2\pi}{\lambda} \right)^4 \left( \frac{n^2 - 1}{n^2 + 2} \right)^2 \left( \frac{d}{2} \right)^6$$

Equation 1 - Scattering intensity for spherical particles as per Rayleigh model

$$\sigma_s = \frac{2\pi^5 d^6}{3 \lambda^4} \left( \frac{n^2 - 1}{n^2 + 2} \right)^2$$
Equation 2 - Scattering cross-section for spherical particles as per Rayleigh model

From these equations, it can be seen that scattering intensity is highly dependent on particle size (sixth order) and the wavelength of light (inverse to the fourth order) as well as symmetric in the forward and reverse directions. Additionally, intensity is related to the index of refraction according to the ratio of quadratic functions which remain well-behaved as long as $n^2$ does not approach -2.

**Plasmonic nanoparticles**

Plasmonic nanoparticles are highly polarizable, metal nanoparticles where the metal-dielectric surface interface gives rise to coherent surface charge oscillations - surface plasmons - under inducement by the coupled oscillations of electromagnetic waves. Unlike bulk metals, relatively large wavelengths can be manifest in the free electron oscillations due to confinement along the metal surface. From the perspective of scattering, the plasmonic particle is thus an induced scatterer, capable of radiating wavelengths of light far greater than the size of the particle itself.

The result of these phenomena is an aberrant absorption and scattering behaviour that varies according to wavelength and exhibits peaks at resonant wavelengths. Mathematically, these points can be seen to correspond to resonance conditions in the equation describing the scattering cross-section:

$$\sigma_{scattering} = \frac{8\pi}{3} k^4 R^6 \left| \frac{\varepsilon_{particle} - \varepsilon_{medium}}{\varepsilon_{particle} + 2\varepsilon_{medium}} \right|^2$$

Equation 3 - Scattering cross-section of a spherical particle in terms of dielectric functions

Resonance thus occurs at the wavelength where $\varepsilon_{particle} + 2\varepsilon_{medium} \to 0$ or, where $\varepsilon_{medium} \approx 1$, when $\varepsilon_{particle}^2 + 2 \to 0$ or, put in optical terms, where $n^2 + 2 \to 0$ (as per Equation 1).

Furthermore, where the metallic dielectric function for a free electron can be expressed as: $\varepsilon_{particle} = 1 - \frac{\omega_p^2}{\omega^2}$, resonance can thus be defined as where $\omega_p^2 = 3\omega^2$.

Of particular interest are particles that exhibit said resonance wavelength, otherwise referred to as a plasmonic peak, at the visible wavelengths that are most relevant to common technologies such as...
microscopy. For that reason, gold and silver dominate as plasmonic nanoparticles given that they exhibit scattering and absorption peaks in the green and violet regions, respectively. Other popular metals include copper, titanium, platinum, and iron whose spherical nanoparticles exhibit plasmonic peaks in the red, deep UV, UV, and IR regions, respectively.

Whereas particle size and shape are central to the aforementioned scattering cross-section, they also define the dielectric function that manifests resonance. Whereas spheres will be the only geometry considered experimentally here, asymmetry in scatterers offer disparate resonant axes; most famously, nanorods exhibit scattering peaks that correspond with resonance along their short and long axes. Size, of course, has a similar effect in defining resonant frequencies: as a spherical plasmonic particle dilates, its plasmon peak redshifts. In practice, this is an invaluable symptom of particle aggregation, where the first sign is often a redshift in scattering.

In terms of differentiability then, the ideal plasmonic scatterer would have an arbitrary plasmonic peak. Instead of being limited to the peak scattering wavelengths resulting from spherical particles of pure materials at various radii, a label particle would ideally have a scattering peak (observable as colour under dark field imaging) that is just as programmable as the receptor that will be targeted.

Gold/Silver alloy nanoparticles (and their synthesis)

(as developed and performed by Sebastien Besner and then David Rioux of the Meunier Lab)

To meet this need for a plurality of labels that were easily differentiable by their scattering behavior, alloy nanoparticles were used as opposed to single-material nanoparticles. Alloys, mixing gold and silver atoms, manifest a Plasmon peak according to their constitution; a particle that is half gold and half silver, for example, expresses peak scattering at a wavelength ≈460 nm, halfway between that of pure gold (≈520 nm) and pure silver (≈400 nm). By controlling the mole fraction of synthetic constituents, the ultimate Plasmon peak can thus be arbitrarily controlled between the limits of pure materials.

Synthesis

The synthesis of alloy nanoparticles begins with the fragmenting of pure metals with a femtosecond (fs) laser, mixing the pure metal fragments in their constituent ratio in a solution of a stabilizing polymer,
and then ablating the bimetallic mixture of fragments with the fs laser such that alloy nanoparticles form with a constitution reflecting the molar ratio of the fragments mixed.

A pulsed Ti:Sapphire laser (Hurricane, Spectra Physics) with a pulse width of 110 fs (FWHM) centered at 800 nm, repetition rate of 1 kHz, laser spot of ≈0.5 mm in diameter, and energy of 500 μJ/pulse is used for ablation. Gold and silver fragments are produced at rates of approximately 0.05 mg/min for silver and 0.2 mg/min for gold, then weighed to their desired amounts by analytical balance, and mixed in a separate glass vial in an excess of Dextran (500kD MW, 1g/L). The mixture was then ablated again, forming the colloidal alloy suspension. High resolution TEM and electron dispersive X-ray analysis <Figure 4> verified that the product nanoparticles were both crystalline and relatively homogeneous in their constitution (corresponding to the initial mole fraction).

As can be seen in Figure 3, the scattering peak for alloy nanoparticles varies as expected, as per the weighted averaged of the Plasmon peaks of the constituent elements.

![Extinction spectra for various constitutions of AgAu alloy NP](image)

*Figure 3 - Extinction spectra for various constitutions of AgAu alloy NP*
**Immunological targeting**

The strategy for targeting cancerous cells was premised on recognizing signatures of cell surface receptors. Whereas all leukocytes express the CD45 receptor, for example, B-cells express CD19 and CD20, and cells associated with chronic lymphocytic leukemia (CLL) further express CD5 and CD23 - CLL cells may thus be defined by this characteristic coexpression. Receptor types are targeted individually by conjugating their corresponding specific antibody to the nanoparticle surface and relying on their natural complimentarity in binding.

**Nanoparticle functionalization**

The chemical functionalization of gold, silver, and alloy nanoparticles allowed them achieve both stability in preparation and application, and immunotargeting for a given cell surface receptor via the conjugation of a specific antibody. Thiol-binding interactions with gold and silver surfaces attached two forms of bifunctional PEG-based tethers that would stabilize the particles and allow for the subsequent EDC/NHS coupling reaction that transformed free acid groups to amine-reactive NHS-esters and ultimately bound the targeting antibody groups. The chemistry underlying these key steps will be discussed as reference for the presented procedure.
Figure 5 - Overall functionalization procedure for plasmonic NPs

**Thiol bonding**

The metal–thiol bond provides a convenient mode of surface functionalization as strong, dative bonds are generated via simple exposure to a free thiolo. The Au/Ag-thiol bonds are particularly strong and will provide the backbone for binding bifunctional PEG chains to the nanoparticle surface.

**EDC/NHS Coupling**

In order to increase the ultimate stability of the label, the immunotargeting agent was covalently bound to the outside of the PEG-coated particle. This was accomplished through EDC/NHS coupling, a strategy that converts free acid (-COOH) groups to primary amine-reactive esters, perfect for crosslinking with antibodies.

The process starts with free acid reacting with EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) to form an unstable O-acylisourea intermediate. This can directly react with a primary amine but overall coupling efficiency is increased dramatically by introducing a second species, N-hydroxysuccinimide (NHS) or its water-soluble sulfonated analogue, Sulfo-NHS. This offers another pathway for the intermediate to avoid hydrolysis [back to an acid] and convert to another primary amine-reactive form, the N-Hydroxysuccinimide Ester (NHS Ester).

Coupling efficiency as a function of the reaction concentrations of EDC and Sulfo-NHS was studied using IR spectroscopy by Sam et al. Using an acid-functionalized porous silicon substrate as a test surface, they deduced reaction products in a semi-quantifiable way from detectable IR bands and described the activation process in terms of competition between three possible products from the O-acylisourea
form, one of which was a terminal, unreactive urea by-product <Figure 6>. They demonstrated not only that a balance was desired between the concentrations of EDC and Sulfo-NHS (too much or too little of either emphasized an undesirable pathway) but that, even in tandem, excess EDC/Sulfo-NHS resulted in the terminal Urea product <Figure 7>. The quantitative conclusions of the study were inapplicable to the radically different surface transport dynamics of a nanoparticle system but the qualitative lessons were invaluable.

Figure 6 - Reaction pathways of free -COOH with EDC/Sulfo-NHS. Reprinted (adapted) with permission from Sam, S. et al. Semiquantitative Study of the EDC/NHS Activation of Acid Terminal Groups at Modified Porous Silicon Surfaces. Langmuir 26, 809–814 (2010). Copyright 2013 American Chemical Society.

Figure 7 - Functionalization outcomes for the reaction of -COOH with various concentrations of EDC/Sulfo-NHS; outcomes defined by overlapping regions of terminal products. Reprinted (adapted) with permission from Sam, S. et al. Semiquantitative Study of the EDC/NHS Activation of Acid Terminal Groups at Modified Porous Silicon Surfaces. Langmuir 26, 809–814 (2010). Copyright 2013 American Chemical Society.

Visible imaging of ANPs
The initial challenge in imaging via dark field microscopy was achieving a signal-to-noise ratio that was suitable for imaging. As with every sensing mechanism, useful imaging was only possible when the signal originating from the intended scattering targets (the signal) was differentiable from background scattering (the noise).

**Sample preparation for imaging**

The initial, continuing, and most mundane challenge in dark field imaging was mitigating background scattering (i.e. reducing the noise in the S/N). Given the low intensity of scattering from nanoparticles (the signal) and the relative size of potential background scatterers (noise), it was critical that the imaging environment be optimized. The noisiest culprits were identified as surface contamination, bulk occlusions/defects, and surface roughness.

**On: Slide Cleaning**

Initial attempts to image under dark field conditions naively followed experience in clinical histology labs, where "pre-cleaned" slides were sufficient for the imaging of stained cell samples. It was immediately apparent that this was insufficient as scattering from latent dust and dirt overwhelmed everything but scattering from cell membranes. It was thus necessary to clean the slide so various methods were attempted in order to establish the easiest method to achieve the best effective surface cleanliness. The upper-bound for cleanliness was effectively established by the quality of the glass, where some density of scattering occlusions would always be present.

Various cleaning steps were attempted in various combinations, namely:

- Rinsing with H2O
- Scrubbing with soap water
- Sonicating in H2O
- Rinsing with acetone
- Sonicating in acetone
- Bathing in Piranha

(Any water used was 18.2MΩ Milli-Q dispensed)

Though, by inspection, it seemed that it was mostly dirt and dust that needed to be removed, acetone was included as a sample organic solvent. It did no better job than soap water in terms of dissolving any organics and left a visible residue that needed to be cleaned, in turn. Ironic but not helpful.
As expected, soaking the slide in Piranha did an excellent job of removing any solids from the slide surface but the slide still showed some residue after a rinsing step with water.

Simply rinsing the slide with water left a significant number of particles as well as residues; scrubbing with soap water tended to remove particulate but residues would remain.

The key to achieving "pristine" surfaces in terms of surface contamination was sonicking the slide in H₂O as the ultimate cleaning step. Slide holders, as used for slide staining in histology labs, were suspended in the sonication bath in order to facilitate the cleaning of many slides at once. Piranha cleaning and scrubbing with soap water were found to be equally effective as ante-pan-ultimate steps, where the slide was always rinsed with water before sonication; scrubbing with soap water was adopted for ease, safety, and cost. The same process was followed for coverslips.

It was further found that "blow drying" the slides by compressed N₂ - rather than allowing them to air dry by evaporation - obviated the drying rings that would result despite the cleanliness of all solutions involved.

**Bulk defects**

Any bulk glass, from IKEA-grade water glasses (borosilicate) to fab-grade fused silica wafers, is guaranteed to have some finite density of defects but the prominence of these inclusions as scatterers vary wildly depending on said density and the type of defects. Various transparent substrates were tested in order to find an ideal material for sample slides, measured in terms of average background scattering:

- **Polystyrene-derivative:** Easily handled and cleaned, cheap, chemically resistant, but exhibited unavoidable bulk scattering sites
- **Soda lime:** Standard slides – there was slight variation between manufacturers but some bulk defects were unavoidable
- **Quartz & Fused silica (SiO₂):** Though they exhibit perfect clarity, cost made them unsuitable for common imaging.
The mounting and sealing procedure made recycling substrates prohibitive so the cost of quartz and fused silica substrates meant that soda lime was the only acceptable option.

**Surface Roughness**

Just as bulk defects act as scattering centers, so do surface features manifest in superficial roughness. Given the shallow depth of focus when imaging at high magnification, only the side of the slide closest to the objective was relevant in terms of background scattering. This face sandwiched the sample along with the coverslip so the offending interface was between the slide and the sample media (and then, similarly, the sample media and the coverslip). In order to mitigate scattering as much as possible, the sample media was chosen so as to minimize the juxtaposition of its index of refraction with that of soda lime glass ($n \approx 1.52$).

**Cell handling**

The handling of cells prior to imaging was essential to maintaining their integrity and minimizing their scattering. Overly vigorous mixing, not maintaining the appropriate aqueous environment, or mounting the sample before fixation could cause the cell to burst; not only does this endanger accurate targeted binding but it leaves a highly scattering residue that distracts from any particle colouration. Handling and, of course, mounting solution makes a dramatic difference in terms of background scattering.

![Figure 8 - Dark field image of patient leukocytes mounted poorly](image_url)
Spectral analysis

In order to analyze the scattering behaviour of particles, the light from the image could be redirected (out the left port) into a spectrometer. For dense collections of particles occupying the entire field of view, this could generate an accurate scattering spectrum but, in any practical application, background scattering from cells would wash out any obvious plasmonic character. In order to be useful, the view would need to be restricted so as to only collect light from the particles of interest; this would require a pinhole aperture, most conveniently placed at the focal plane of the microscope exiting the microscope.

Figure 9 - Dark field image of patient leukocytes handled appropriately and mounted with CFM-1

Figure 10 - Dark field image of a dense collection of 60nm Au NPs
Figure 11 - Spectrum corresponding to scattering from dense collection of 60 nm Au NPs ($\lambda_p$≈550 nm)

**Equipment:**

**Optical microscope**
- Microscope: Nikon Eclipse TE2000-U
- Light source: Nikon Halogen (LHS-H100P-1) 12V 100W
- Illuminator controller/Power Supply: Nikon TE2-PS 100W
- Condenser: Nikon Dark Field Condenser (Dry, NA: 0.95-0.80)
- Objective: CFI Plan Fluor ELWD 60x (NA: 0.70, WD: 2.1-1.5 mm, Correction Collar: 0.5-1.5 mm)
- Imaging CCD: Nikon Digital Sight DS-Fi1
- CCD Controller: Nikon Digital Sight DS-U2
- Capture software: NIS Elements

**Functionalization/Sample preparation**
All water used was as dispensed by a Milli-Q filtration system, rated at 18.2MΩ

- Ted Pella ([http://www.tedpella.com](http://www.tedpella.com)):
  - 60nm Au NPs (15708-6)

  - HS-C2H4-CNH-PEG-O-C3H6-COOH (13 5000-4-32)
  - CH3O-PEG-SH (12 5000-40)

- Fischer Scientific ([http://www.ecat.fishersci.ca](http://www.ecat.fishersci.ca)):
  - Sulfo-NHS (PI24510)
  - EDC (PI22980)

- Beckman Coulter ([http://www.coulterflow.com/](http://www.coulterflow.com/)):
  - CD45 Antibodies - Monoclonal, Ros220 + ALB12, Anti-human (IM1916)
  - CD19 Antibodies - Monoclonal, J3-119, Anti-human (IM1313)

- Millipore ([http://www.millipore.com](http://www.millipore.com)):
  - 100 kD Centrifugal Filters - Amicon Ultra-0.5, Ultracel-100 Membrane

- Electron Microscopy Sciences ([http://www.emsdiasum.com](http://www.emsdiasum.com)):
Entellan Sealant (14800)
CFM-1 Mountant Solution (17980-10)

Functionalization procedure

Optimizing for all the aforementioned factors, the following procedure was developed for nanoparticle functionalization, cell incubation, and imaging under dark field conditions. Where particles were imaged outside of cell targeting studies, the particle solution was ultimately resuspended in CFM-1 and 8 μL of particle solution was applied to the slide and mounted as described.

Solutions used:
20 μM mPEG (1mL H2O / 100μg of CH3O-PEG-SH)
20 μM COOH-PEG (1mL H2O / 100μg of HS-C2H4-CONH-PEG-O-C3H6-COOH)
Sulfo-NHS: 40 mg/ml H2O
EDC: 40 mg/ml H2O
Filtered Antibodies:
   Add 200 μL of antibodies to 100 kD filter
   Spin @ 14 kG for 15 mins
   Invert filter, spin @ 1 kG for 3 mins

PEGylate nanoparticles
Per 1 ml Au NPs,
Add 25 μL of mPEG
Stir for 15 minutes
Add 800 μL of cPEG
Stir for 30 minutes
Centrifuge at 10 000 g for 10 minutes (#1)
Remove supernatant
Resuspend in H2O
Centrifuge at 10 000 g for 10 minutes (#2)
Remove supernatant
Resuspend in H2O
Centrifuge at 10 000 g for 10 minutes (#3)
Remove supernatant
Resuspend in H2O

EDC/NHS activation
Add 5 μL of EDC
Add 5 μL of Sulfo-NHS
Stir for 15 minutes
Centrifuge at 10 000 g for 10 minutes (#1)
Remove supernatant
Resuspend in H2O
Centrifuge at 10 000 g for 10 minutes (#2)
Remove supernatant
Resuspend in H2O
Conjugate antibodies to nanoparticles
Add filtered Antibody solution to NPs
Let sit for 4 hours @ RT
Centrifuge at 10 000 g for 10 minutes (#1)
Remove supernatant
Resuspend in H2O
Centrifuge at 10 000 g for 10 minutes (#2)
Remove supernatant
Resuspend in H2O
Centrifuge at 10 000 g for 10 minutes (#3)
Remove supernatant
Resuspend in PBS + FCS

Incubate NPs with cells
Combine NPs & cells, vortex lightly
Maintain movement for 2 hours @ RT
Centrifuge @ 1 kG for 5 minutes (#1)
Remove supernatant
Resuspend in PBS w/1% FCS
Centrifuge @ 1 kG for 5 minutes (#2)
Remove supernatant
Resuspend in PBS w/1% FCS
Centrifuge @ 1 kG for 5 minutes (#3)
Remove supernatant
Add 100 μL of paraformaldehyde, vortex lightly
Let sit for 15 minutes
Centrifuge @ 1 kG for 5 minutes
Remove supernatant
Resuspend in PBS w/1% FCS

Mounting sample for microscopy
Cytospin on slide
Add drop of CFM-1, lay coverslip
Add Entellen to corners
Let set for 10 minutes
Seal edges with Entellen

Experimental results

Evidence of targeting (60 nm Au NPs): Imaging
Anti-CD45 conjugated 60 nm Au nanoparticles were incubated with patient cells and imaged as described. For contrast, a negative trial was performed in parallel where functionalization forked after EDC/Sulfo-NHS activation and no immunotargeting agent was added.
Representative results from the negative trial can be seen in <Figure 12>. Sporadic particles are visible in the background but there seems to be no selection for the cell surface.

![Figure 12 - Dark field image of patient leukocytes incubated with untargeted Au NPs (-)](image)

Representative results from the positive, targeted trial can be seen in <Figure 13>. Particles are clearly visible along the surface of the cells. Aggregation is evidenced by a shift in the scattering colouration; though some green scattering (corresponding to the Plasmon peak of an isolated particle) is visible, the majority is golden-orange or even white.

![Figure 13 - Dark field image of patient leukocytes incubated with targeted Au-CD45 NPs (+)](image)
Evidence of targeting (60 nm Au NPs): Flow cytometry

Targeting was further verified using flow cytometry, a clinical tool that probes the fluorescence and scattering of cells as they pass in a controlled flow. Binding efficiencies were tested by incubation with cell sample and post-staining with fluorescent secondary anti-MsIgG1 antibody. Edward Parker of the Wang Lab ran the tests.

To establish a negative reference, the first target was untargeted cells. As expected, the secondary antibodies had no opportunity for binding <Figure 14>.

As a positive reference, cells subjected to CD45 antibodies were stained. Leukocytes universally present CD45 receptors so, as expected, the fluorescent secondary antibodies bound in great numbers <Figure 15>.
As a sanity check, the same positive reference trial was performed with filtered antibodies. It had no impact on signal strength <Figure 16>.

Finally, patient cells were incubated with anti-CD45-targeted nanoparticles then stained. The signal is highly positive, indicating the binding of secondary antibodies and thus implying the presence of bound targeted particles <Figure 17>.
Evidence of differentiability (AgAu alloy NPs)

The prerequisite step in using alloy nanoparticles as labels under dark field imaging was ensuring their differentiability in scattering. Particles were prepared and imaged as previously outlined.

Pure silver particles, synthesized by the Meunier ablation technique, were imaged (Figure 18). Representing the short wavelength extreme ($\lambda_p \approx 400$ nm), they exhibited a sharp, bright blue with a violet tinge; the apparent bias toward red scattering character was an artifact of the light source which was heavily-weighted toward red ($\lambda_{600}/\lambda_{400} \approx 10$).
Ag$_{0.5}$Au$_{0.5}$ alloy nanoparticles were imaged similarly, offering a turquoise, greenish colour <Figure 19>. Again the lamp bias was evident but they were clearly differentiable from the pure Ag particles.

![Figure 19 - Dark field image of ablated Ag$_{0.5}$Au$_{0.5}$ NPs](image)

Finally, pure gold nanoparticles, generated as always via ablation, were imaged <Figure 20>. As expected, they demonstrated their characteristic green scattering, though noticeably bluer than the 60nm particles imaged previously.

![Figure 20 - Dark field image of ablated Au NPs](image)
With the imaging procedure refined sufficiently to image the smaller alloy nanoparticles, the three sample species offered scattering behaviour that was easily differentiable under dark field imaging. It is possible that more distinct label colours could have been derived from the Ag-Au range but three reference types were sufficient in order to demonstrate the proof of concept for targeting.

**Evidence of targeting (AgAu alloy NPs)**

In order to demonstrate targeted binding of the immunofunctionalized alloy nanoparticles, anti-CD45 programmed particles were incubated with patient leukocytes, mounted, and imaged as before. The same set of particles that were assessed for their differentiability (Ag, Ag\textsubscript{0.5}Au\textsubscript{0.5}, Au) were used for targeting studies.

In order to establish a negative reference, cells were incubated with untargeted particles then imaged under dark field <Figure 21>. As hoped for, there was no sign of bound particles.

![Figure 21 - Dark field image of patient cells incubated with untargeted Au NPs](image)

Anti-CD45 targeted Au particles were incubated with patient leukocytes and imaged under dark field <Figure 22>. Bound particles were visible but exclusively in large aggregates with a colouration that was almost entirely orange and red.
Anti-CD45 targeted Ag₀.₅Au₀.₅ particles were incubated with patient leukocytes and imaged under dark field microscopy. Again, particles were visible but only in very large collections; scattering colour ranged from green to orange.

Anti-CD45 targeted Ag particles were incubated with patient leukocytes and imaged under dark field microscopy. As before, particles were only visible in substantial groups. Colour appeared unchanged but, given the lack of violet light available from the illumination source, an aggregation-induced Plasmon shift on the order of what was seen in the other two samples would not have been apparent.
Figure 24 - Dark field image of patient cells incubated with Ag-CD45 NPs

Discussion:
These experiments confirmed key elements of a functional proof of concept technology. The platform’s goals were accomplished to varying degrees; whereas some results represent intrinsic limitations of the platform, others merely point to the more challenging aspects yet to be solved. The extent to which core goals, as established in the prior discussion of motivation, were accomplished will be assessed and discussed in terms of future work.

The differentiability of alloy nanoparticles according to their constitution
Toward the prime application in clinical pathology, label multiplexing, the ability to distinguish a plurality of different labels (each corresponding to a different receptor) in a single image, was a key metric. In addition to the sheer number of labels, however, an important qualitative distinction arose in practice, namely how differentiation could be achieved. Spectroscopic differentiation, whereby the label’s “identity” would be assessed as per a spectroscopic analysis of its scattering, was seen as the lower bar; despite offering much more specificity – a precise description of the scattering peak, it would require additional equipment in order to isolate the scattering from only the labels in question, materials outside of what is typically available in light microscopy: a spectrometer, confocal/pinhole setup, and the optics to align the two. Furthermore, though this method would offer the appearance of specificity in the abstract, in practice, it was impossible to isolate the scattering from labels alone, introducing variability in the signature of labels that mitigated any apparent advantages. Even under ideal
conditions, it would still pale in comparison to another spectroscopic technique such as SERS which offers a far sharper scattering signature.

The standard considered was thus visible differentiability, whether directly by eye or as represented by in CCD images. Though apparently sacrificing the prospect of multiplexing amongst a greater number of labels, this approach would offer the tantalizing opportunity of identifying cell receptors by eye alone, much more in-line with the current clinical practice of judging cell morphology. For further analysis, colouration could be defined in more quantitative terms from images, relying on image processing software. Toward this goal, experiments were clearly successful in differentiating between the three sample alloy nanoparticle constitutions used (Ag, Ag$_{0.5}$Au$_{0.5}$, and Au). As synthetic techniques improve to generate alloy particles of increased size (stronger signal) and lower polydispersity (sharper colour), and given a light source better tuned to violet, the number of Ag/Au particles differentiable by eye could easily number four or five. Furthermore, if Cu were integrated as a constituent metal as seen in other experiments from the Meunier group, the library of differentiable labels could range the entire visible spectrum, covering violet to green with AgAu alloys and green to red with AuCu alloys.

**That alloy nanoparticles can be coimaged alongside cells**

The ability to image nanoparticles alongside cells under dark field, though initially daunting, was a matter of signal-to-noise. Optimization in terms of imaging, mounting, and cell handling drastically improved visibility as first verified with 60 nm Au nanoparticles. Given particle scattering’s dramatic size dependence (proportional to $D^6$), alloy nanoparticles were a significant challenge but, with larger particles (≈30 nm), the ultimate process proved sufficient. Increases in particle size will further minimize this challenge.

**That immunotargeting by nanoparticles is possible [while retaining signal integrity]**

The label’s core competency was its ability to selectively bind to cell surface receptors. Starting with 60 nm Au particles, targeting was demonstrated with anti-CD45. Juxtaposing negative and positive trials, there was a clear lack of any binding in the former case (Au-mPEG/cPEG) and significant surface binding of the particles to the ubiquitous CD45 receptor in the latter case (Au-PEG-CD45). Targeting was verified in flow cytometry, applying fluorescent secondary antibodies (anti-anti-CD45).
Functionalizing alloy nanoparticles was complicated by their more delicate handling but targeting studies showed initial success in binding. Compared to negative trials, where there was no evidence of nanoparticles that were not functionalized, particles conjugated with anti-CD45 were found bound to cell samples. Unlike the previous targeting trials, however, particles were only found bound in large aggregates. This would imply either that the functionalization or incubation process induced aggregation, or that cell-binding encouraged the aggregation process. Particles were imaged in isolation once functionalized (but prior to incubation) to see if aggregation was occurring before application, but dark field imaging showed no evidence of a red-shift in scattering colour. No further studies were performed but the next step would have been to explore if the aggregation was solution-induced by transferring functionalized particles into a solution emulating a cell solution, PBS with 1% FCS. If aggregation was not found to be solution-induced, one might consider a non-specific, non-binding interaction between particles and cells by functionalizing particles with an antibody corresponding to a receptor not found on the cell and incubating them with patient cells. If aggregation was still not induced, the behaviour would have to be explained by cell receptor binding.

In addition to targeted binding, it was also critical that the label maintain its scattering colour (ie not aggregate to such an extent that there is a substantial shift in the Plasmon peak). Again, behaviour in experiments was promising but non-ideal. In their bound, aggregate forms, the Ag particles maintained their colour whereas the Au and Ag$_{0.5}$Au$_{0.5}$ particles should significant shifts; Au particles, normally deep green, ranged from deep green to orange and light-red whereas Ag$_{0.5}$Au$_{0.5}$ particles, normally bluish-light green, ranged from the expected light green to a dark green and some orange. This shift in scattering colour was exactly what would be expected given such significant aggregation in binding.
Quantifying densely-bound SERS labels

Motivation and Strategy:
As the prospect of using SERS particles as cellular labels rises, one of the challenges in their clinical application is the quantification of particles in areas of high-density. Proximate particles are known to have a coupling effect that amplifies their surrounding electric fields resulting in an exponential (rather than linear) rise in scattering intensity as particles are added within an interacting range of each other. This phenomenon can be a boon in terms of signal amplification, but it makes counting particles within this interacting length scale a substantial challenge as scattering intensity becomes a function of not only the number of labels but their position. In order for SERS particles to be useful as cell surface receptor targeting labels, it is important to not only sense the presence of the label (and thus a corresponding receptor) but quantify them. This would not only offer information about the binding dynamics of particles but the density and [potentially dynamic] position of receptors on cells. Moreover, it would allow for a calibration between current clinical diagnostic thresholds related to receptor density and those that would need to evolve based on SERS.

In order to address this challenge, two experimental platforms were designed to exemplify the scattering of interacting SERS particles in pairs and groups. The first used pairs of receptor anchor sites at precise distances from each other to establish an experimental relationship between the intensity of SERS scattering, $I_{\text{SERS}}$, and interparticle separation, $d_{ip}$. The second platform related scattering intensity over a large potential binding area to an increasing concentration of bound SERS particles; by measuring $I_{\text{SERS}}$ as varying concentrations of integrin receptors are bound the surface then saturated with RGD-targeted SERS labels, the various regimes of scattering behaviour – defined by the mean distance between bound particles – will be elucidated.

From the distance-dependence study, a calibration curve will offer $I_{\text{SERS}}$ v $d_{ip}$; two key figures will be visible: the critical interparticle distance at which scattering is maximized, $d_{\text{crit}}$, and the interaction distance at which interparticle coupling effects become germane, $d_i$. From the second platform, the density regime of linear scattering response will be defined, and the mean interparticle distance, $\overline{d_{ip}}$, at the transition from linear to exponential regime will imply $d_i$. Finally, combining the distance-dependence relationship with density calculations, it should be possible to interpolate the nanoparticle population in the medium density, non-linear regime given knowledge of the sampling area.
Binding will rely on a cell surface receptor-like binding between substrate-bound α5β3 integrin receptors and SERS Au nanoparticles conjugated with complimentary RGD peptides. SERS particles are thus programmed by custom synthesized bifunctional HS-PEG-RGD whereas integrin receptors were fixed to the substrate via the familiar EDC/Sulfo-NHS chemistry; HS-PEG-COOH was bound to platinum anchor sites via thiol bonds and the free acid at the opposite end was reacted with EDC/Sulfo-NHS in order to then bind to the receptor. By saturating substrate bound receptors with targeted SERS labels, the patterning of Platinum anchor sites effectively acts as a proxy for the ultimate placement of SERS labels. This allows for the study of scattering from SERS particles in arbitrary arrangements, a control normally unavailable through more direct techniques.

**Past work**

Though theoretical studies have long simulated the plasmonic properties of various shapes, sizes, and materials, there has been increasing study of their performance as SERS-type scatterers, namely toward maximizing scattering intensity from particles and thus their utility as labels. One of the most popular approaches has to been to leverage the local electric field enhancement effect of particles by grouping them closely in a controlled manner and harnessing their mutually reinforcing amplification effect. This phenomena has been studied extensively in terms of the simplest coupling geometry, dimers; theoretical studies, based on fundamental electromagnetic simulations, have established expectations for particle pairs and there have been some complimentary experimental studies. Notable in these experimental studies is the lack of control and regularity in terms of particle placement and character. There have also been exciting sensing platforms developed that are premised on shifts to scattering behaviour (resonance frequency) upon the binding of a second plasmonic particle but they were less unconcerned with general relationships. Studies on high-density groupings of SERS particles, though promising in terms of developing superlative scatterers, have largely been focused on harnessing the cumulative amplifying effect of aggregation rather than deconvolving population and position.

**Substrate patterning**

**“FormatFlow”: From software to software to hardware**

The lithography systems used to pattern in fabrication were dependent on hardware specific instruction files generated by intermediate conversion software, LayoutBEAMER. By using configuration files
specific to the lithographic hardware and specifying various parameters for beam plotting geometry, CAD files were converted into the requisite instructions specifying shot placement by layer for interpretation by the lithography system’s controller. Pattern exposure was ultimately manifest by run files that specified the organization and beam dosage of the specified layers.

CAD was done using L-Edit Layout Editor software from Tanner EDA (http://www.tannereda.com/l-edit-pro). Designs beginning in the proprietary .tdb project format were exported to .GDSII, a standard format for integrated circuit layout data. Layered layout design could be implemented either through the explicit drawing of objects, one or two-dimensional arrayed instancing of objects, or the scripting of instantiation through programmatic functions (in pseudo-C syntax). Standard arraying was sufficient for the repetition of identical objects (substrate chips) but variations within chips (subarray indices and the incrementing separation distances within anchor site pairs) required dynamic instantiation. The programmatic approach will be described further per particular chip design.

**Dynamic functions: Stencils and Pairs**

Though simple geometries (rectangles, circles, triangles) could be generated automatically through the L-edit software, more complex forms were desired as both macro-indices (arrows pointing to micro/nanoscale features on macro substrate) and sub-array indices (numbers). In order to generate them, functions were written to draw the desired shape with the desired orientation at the desired position. These functions were essential in the ultimate scripting of dynamically instantiated sub-array indices, where features evolved with the array.

More important than helpful markers, however, was the ability to define pairs of anchor sites where the separation distance was defined dynamically. A function was written that drew the pair centered at a given location with a parameterized separation distance [that could be then be incremented according to array indices].

**On: Patterning**

The general strategy in lithographic patterning is that a lithographic system patterns a resist which, once developed, exposes the intended image on the substrate, and, upon the indiscriminate application of the deposited material, manifests a pattern. Lithography can be implemented by any means - be it a palette knife in print, UV lamp in photolithography, or electron beam in e-beam lithography – and,
especially when it comes to chemical rather than mechanical exposure, the patterned resist can represent the positive or negative image. The pattern is developed, leaving either the resist that was exposed or left unexposed, and then the ultimate deposition material is distributed over the entire substrate. Where no resist remains, the material is deposited directly on the substrate and, when the resist is removed, all that remains is this image.

![Diagram of lithographic process](image)

**Figure 25 – Schematic of lithographic process**

*The Surface*

In choosing a functional material for use as a basis for attaching binding sites, there were three key factors to consider: ease of use in fabrication, potential for functionalization, and complexity in SERS signal analysis.

*Fabrication*

This was a key initial limitation as a limited number of materials are compatible with the full range of nanofabrication processes. Selection began with this short list of materials that could be sculpted with nanometre precision via electron beam lithography and deposition.

*Functional*
Though platinum’s bond with thiols is not nearly as strong as that with gold or silver, it is still suitable for functionalization with thiolated PEG\textsuperscript{65,66}. By using the previously discussed bifunctional carboxylic acid and thiol-terminated PEG polymer (cPEG: HS-PEG-COOH) and EDC/NHS cross-linking chemistry, Integrin receptors can be covalently bound to the surface for use as cell receptor-like binding sites for SERS nanoparticle targeting.

**Coupling**

In addition to being functionally capable, it was important that the surface material would not confuse scattering measurements. Though fabrication and functionalization would have been trivial with gold, Plasmon coupling between SERS nanoparticles and the surface would have conflated the scattering signal intensity. Titanium, used as an adhesion layer, and platinum have particularly poor plasmonic character with resonance in the mid-IR\textsuperscript{67,68}, far from any wavelength resonant in the gold nanoparticles.

**Functionalization procedure**

The anchoring material was specifically chosen in order to allow for the same bifunctional PEG-based binding that was used in both nanoparticle systems. The profoundly dissimilar transport dynamics associated with the surface functionalization of planar substrates (as opposed to nanoparticles that exhibit vastly increased surface area\textsuperscript{69}) forced significant changes to the functionalization chemistry but the same strategy could be employed, adjusted for heightened cleaning requirements and significantly longer incubation periods.

- Bath in Piranha (3:1 H\textsubscript{2}SO\textsubscript{4}:H\textsubscript{2}O\textsubscript{2}) for 90 s
- Rinse with water
- Bath in PEG solution for 12 hours
- Rinse with water
- Bath in 1:1 EDC/NHS (5mM) for 30 min
- Rinse with water
- Bath in Integrin solution for 6 hours
- Rinse with water

**Targeting System**

Cell-type binding was represented by Au-PEG-RGD binding with Integrin-PEG-Pt/Ti surface receptors.

**SERS label functionalization**
The functionalization process for SERS particles was very similar to that of labels for imaging under dark field microscopy. Though the optical process observed was fundamentally different, the functionalization followed the same core principles of using bifunctional PEG for stabilizing the particle and offering a free acid group to ultimately crosslink to targeting antibodies via EDC/NHS coupling. The key addition preceded the aforementioned functionalization chemistry: the electrostatic adhesion of Raman reporter molecules, rendering them SERS particles.

In the presented sample procedure, as prepared and performed by Glenda Sun of the Walker Lab, Malachite Green is used as Raman reporter.

Prepare 4µM solution of MGITC
Prepare 20µM HS-PEG-CH3 (mPEG)
Stir 3mL of Au NPs
Add 0.5 mL of MGITC drop-by-drop
Stir for 15 minutes
Add 195µL of mPEG solution drop-by-drop
Stir for 20 minutes.
Centrifuge @ 10,000 rpm for 5 min, remove supernatant and resuspend in water. Repeat once.
Resuspend to 2ml

Prepare 1µM HS-PEG-COOH (cPEG)
Add 430µL of cPEG drop-by-drop
Stir for 15 minutes
Add 1.6mL of mPEG
Stir for 10 min
Centrifuge @10,000 rpm for 5 min
Remove supernatant and resuspend in water. Repeat once.
Prepare EDC (40mg/mL), Sulfo-NHS (110mg/mL)
Add 5µL of EDC
Add 5µL of Sulfo-NHS
Stir for 15 min
Clean by centrifugation (x2)
Resuspend in water
Add 11.2 nmol of antibodies
Let sit @RT for 2 hrs
Store reaction overnight
Clean via centrifugation

Particle Imaging

Though all the primary measurements planned for the two platforms were focused on the intensity of Raman scattering, multimodal imaging was desired in order to first verify the assumption that binding
anchor sites were saturated by SERS labels and, secondly, help model particle coupling interactions at sub-wavelength lengthscales, relating \( I_{\text{SERS}} \) with direct data on particle positions at high density. This was only possible where various modes of imaging/sensing could be collocated, made possible by the careful indexing of the anchor sites on the two platforms.

Two modes of imaging/sensing were planned: Raman scattering and Scanning Electron Microscopy (SEM). In addition to indexing, this concept of multimodal imaging would only be useful if data capture (and correlation) could be automated.

**Confocal raman microscopy**

Raman scattering was probed with a commercial confocal Raman microscope (Renishaw InVia) offering a field of view of \( \approx 1\mu m \times 1\mu m \), a minimum step size of 100nm ± 10nm, and repeatability in positioning of \( \approx 300\text{nm} \). These figures were critical in designing substrates that could be unambiguously probed automatically in order to generate statistical sets of data. Three laser lines were available for probing; sample Raman spectra collected over an integration time of 1s were collected with each of the laser lines as a proof of concept <Figure 26>.

![SERS Scattering from Au(MalGr)-PEG-RGD](image)

**Figure 26 - SERS spectra from exciting a sample label at available wavelengths**

**Scanning Electron Microscopy**
The simplest method to image the experimental substrates with nanoscale resolution was to use scanning electron microscopy to “directly” resolve nanoparticles. The system, however, provided a few challenges relative to traditional EM samples: a large imaging area, an insulating substrate, high resolution targets, and a desire for substrate reusability.

![Figure 27 - SEM image (VP mode) of 60nm Au NPs](image)

Imaging capability was demonstrated and could be used to verify the concept of receptor saturation by assessing a representative sample. To be more broadly useful, however, automation would be required, imaging individual views over the entire anchor site and stitching the results together to form a meta-image encompassing the entire 100 μm x 100 μm area. This is a standard function of the system used and a simple task of mapping scanning over the chip. The greater challenge would be in the automatic interpreting of this meta-image, as will be discussed further.
Anchor site pair platform: SERS labels at a distance

Fabrication Summary

Anchor pairs (electron beam lithography) – layer #1
A freshly cleaned wafer was coated with two layers of PMMA resist (495K then 950K); a lower density lower level results in a slight undercut that facilitates the eventual lift-off process. A conductive layer of Aluminum was deposited on top of the resist to allow for charge dissipation [without being thick enough to hinder high resolution patterning]. The pattern was expressed on the primed substrate via the electron beam lithography system, the Aluminum coating was etched, and the resist was developed. The substrate was cleaned via Reactive Ion Etching (“descum”) and then 10nm of Titanium (adhesion layer) and Platinum (functional layer) were evaporated overttop via an electron beam evaporator. Remaining resist was dissolved (“lift-off”) in methylene chloride bath, leaving metalized pattern on substrate.

Indices (photolithography) – layer #2
The wafer is cleaned via Reactive Ion Etching and primed for resist in a HMDS vapour oven. SPR220 photoresist is spun on, baked, and exposed in a contact photolithography system. The exposed resist is baked again, allowed to rest, then developed. The exposed pattern is etched into the substrate via Reactive Ion Etching under CF4 gas, and the remaining resist is stripped in a bath.

Packaging
A thick layer of photoresist is spun onto the patterned face of the wafer and baked. The opposite face is sealed against plastic backing, and the wafer is diced into chips.
<table>
<thead>
<tr>
<th>#</th>
<th>Step</th>
<th>Tool</th>
<th>Recipe/Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Proximity correct e beam pattern</td>
<td>LayoutBEAMER</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Clean wafer</td>
<td>e beam spinner</td>
<td>Crossfade from Acetone to IPA</td>
</tr>
<tr>
<td>2</td>
<td>Spin e beam resist #1</td>
<td>e beam spinner</td>
<td>495K PMMA (2% in Anisole) @ 4000 rpm for 2 min</td>
</tr>
<tr>
<td>3</td>
<td>Bake resist #1</td>
<td>Hot plate</td>
<td>15 min @ 170 deg C</td>
</tr>
<tr>
<td>4</td>
<td>Spin e beam resist #2</td>
<td>e beam spinner</td>
<td>950K PMMA (2% in MIBK) @ 6000 rpm for 2 min</td>
</tr>
<tr>
<td>5</td>
<td>Bake resist #2</td>
<td>Hot plate</td>
<td>15 min @ 170 deg C</td>
</tr>
<tr>
<td>6</td>
<td>Measure film thickness</td>
<td>Filmetrics film measurement system</td>
<td>PMMA on Si</td>
</tr>
<tr>
<td>7</td>
<td>Evaporate Al</td>
<td>SCCA e beam evaporator</td>
<td>10 nm</td>
</tr>
<tr>
<td>8</td>
<td>e beam exposure</td>
<td>JEOL 9300</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Remove Al</td>
<td>General chemistry acid hood</td>
<td>Soak for 30s, rinse w/DI water</td>
</tr>
<tr>
<td>10</td>
<td>Develop</td>
<td>e beam resist hood</td>
<td>Soak in MIBK:IPA (1:3) for 60s, Rinse with IPA, Blow dry w/N2</td>
</tr>
<tr>
<td>11</td>
<td>Descum</td>
<td>Oxford 80 #1</td>
<td>Argon descum: Etch for 15 sec w/75W forward power</td>
</tr>
<tr>
<td>12</td>
<td>Deposit metals</td>
<td>SCCA e beam evaporator</td>
<td>10 nm Ti; 10 nm Pt</td>
</tr>
<tr>
<td>13</td>
<td>Lift off</td>
<td>e beam resist hood</td>
<td>Soak in Methylene Chloride until release</td>
</tr>
<tr>
<td>14</td>
<td>Inspect</td>
<td>Zeiss SEM</td>
<td>VP mode</td>
</tr>
<tr>
<td>15</td>
<td>Pattern mask</td>
<td>Heidelberg</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Develop mask and etch chrome</td>
<td>Hamatech-Steag Mask Processor</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Strip resist from mask</td>
<td>Resist Hot Strip Bath</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Clean wafer</td>
<td>Oxford 80 #1</td>
<td>60s (O_2@150W)</td>
</tr>
<tr>
<td>19</td>
<td>Prime wafer</td>
<td>YES oven</td>
<td>HMDS vapor (program 0, 30 minutes)</td>
</tr>
<tr>
<td>Step</td>
<td>Process</td>
<td>Equipment/Condition</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>---------</td>
<td>---------------------</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Spin SPR220</td>
<td>Photolithography spinner</td>
<td>30s @ 2k rpm</td>
</tr>
<tr>
<td>21</td>
<td>Bake</td>
<td>Proximity oven</td>
<td>240s @ 115 deg C</td>
</tr>
<tr>
<td>22</td>
<td>Contact lithography</td>
<td>MA6 contact aligner</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Post-bake</td>
<td>Proximity oven</td>
<td>240s @ 115 deg C</td>
</tr>
<tr>
<td>24</td>
<td>Let sit</td>
<td></td>
<td>15 mins</td>
</tr>
<tr>
<td>25</td>
<td>Develop photoresist</td>
<td>Hamatech-Steag Wafer Processor</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Inspect index mark pattern</td>
<td>Optical microscope</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Etch indices</td>
<td>Oxford 100</td>
<td>CF₄ for 10-12 min</td>
</tr>
<tr>
<td>28</td>
<td>Strip photoresist</td>
<td>Shipley 1165 bath</td>
<td>30 min</td>
</tr>
<tr>
<td>29</td>
<td>Spin S1813</td>
<td>Photoresist spinner</td>
<td>30s @ 4000 rpm</td>
</tr>
<tr>
<td>30</td>
<td>Bake S1813</td>
<td>Hot plate</td>
<td>60s @ 90 deg C</td>
</tr>
<tr>
<td>31</td>
<td>Dice</td>
<td>Wafer dicer</td>
<td>All-purpose blade</td>
</tr>
</tbody>
</table>

Table 1 - Fabrication procedure for the anchor site pair platform

(see Appendix I: Lithography tools for tool details)
Fabrication Details

CAD:
Layer #1: Anchor sites (deposited in Ti/Pt)
Layer #2: Photolithographic indices (etched by CF₄)

The 4” wafer was subdivided into a 10 × 10 array of identical ¼” × ¼” chips. Chips featured a 6x6 array where each element was a 28x28 sub-array of anchor site pairs at a given separation distance (Layer #1). Starting at 55nm, centre-to-centre separation distance between anchor site pairs was incremented by 5nm between sub arrays. Elements of both the meta-array and sub-arrays were labeled by indices representing the anchor site separation distance in nm (55 to 210) and two dimensional position within the sub-array (01-01 to 28-28), respectively (Layer #2).

Figure 28 - Wafer-level image of CAD for anchor site pair platform

Figure 29 - Chip-level image of CAD for anchor site pair platform
Instantiation was developed hierarchically: the chip array was defined, each chip instantiated the array where each element represented a different separation distance (complete with index and markers), and each element instantiated its own array of site pairs (complete with indices). Index parameters throughout this hierarchical instantiation allowed for dynamic definitions.
Step 0
The machine instructions generated for the pattern to be written by electron beam lithography is analyzed in LayoutBEAMER software in order to correct for proximity effects.

Step 1
The wafer is cleaned by spinning it rapidly and spraying it with acetone then IPA, never allowing it to dry in between.

Step 2
The wafer is coated with low density PMMA (495K): it is fixed to the spinner by suction, the solution is pooled in the center of the wafer, and the wafer is quickly accelerated to 4000 rpm, at which speed it spins for 2 min. This develops an approximately 47 nm thick layer which will act to undercut the overlayer of PMMA, as the lower density layer will expose more readily.

Step 3
The wafer is transferred to general hot plate held at 170 deg C, where it sits for 15 min. It then rests on a cooling block for 60s so it can return to room temperature.

Step 4
The wafer is coated with high density PMMA (950K): it is fixed to the spinner by suction, the solution is pooled in the center of the wafer, and the wafer is quickly accelerated to 6000 rpm, at which speed it spins for 2 min. This develops an approximately 30 nm thick layer which will act as a nominal over-layer buffer for the more easily exposed resist below.

Step 5
The wafer is transferred to general hot plate held at 170 deg C, where it sits for 15 min. It then rests on a cooling block for 60s so it can return to room temperature.

Step 6
PMMA film thickness is not only highly variable depending on the accuracy with which the film was spun but accuracy in film thickness is critical to having a predictable outcome in electron beam patterning due to the desired feature size and precision in spacing. Accordingly, film thickness was evaluated before
patterning was done using the Filmetrics film measurement system. If a significant deviation from the expected thicknesses was found, the wafer was cleaned and resists were spun again.

**Step 7**
In order to pattern via electron beam lithography without fear of charging, a 10 nm charge dissipation layer of aluminum is deposited on top of the resist.

**Step 8**
The pattern is written directly via electron beam lithography.

**Step 9**
The Aluminum layer is etched by soaking the wafer for 30s in a standard aluminum etch. The wafer is then rinsed with DI water.

**Step 10**
The pattern is developed by soaking the wafer in a 1:3 mixture of MIBK:IPA for 60s. This dissolves the exposed resist, leaving the patterned substrate exposed. The wafer is rinsed with IPA and blown dry with Nitrogen.

**Step 11**
Any remaining residue on the wafer surface is removed by Reactive Ion Etching under Argon plasma for 15s (75W forward power).

**Step 12**
The surface is metalized in an electron beam evaporator under ultra high vacuum. First, a 10nm adhesion layer of titanium is applied, followed by the 10nm functional layer of platinum. Platinum evaporation proceeds slowly to avoid any spattering and, once deposited, the substrate must be allowed to cool gradually to avoid any cracking under thermal contraction.

**Step 13**
The remaining (unexposed) resist is dissolved by bathing the substrate in methylene chloride for 2+ hours (until metalized layer has visibly “lifted off”), leaving the exposed area of the substrate (the
pattern) metalized. Rinse with water to fully release any lingering metal (very necessary) and blow dry with Nitrogen gas.

**Step 14**
Substrate is inspected under Variable Pressure mode, allowing for imaging without depositing the conductive layer normally required for charge dissipation.

**Step 15**
A standard chrome photolithography mask is patterned by a Heidelberg direct laser pattern writer. A laser writing system, which exposes each mask pixel individually, is chosen over a selective geometric exposure system, such as a PG Mask Writer, given the high total writing area.

**Step 16**
The mask is developed in the automatic Mask Processor, leaving a standard chrome photolithographic mask for exposure.

**Step 17**
Remaining resist is stripped from the mask in a hot solvent bath.

**Step 18**
Any remaining residue on the wafer surface is removed by Reactive Ion Etching under an Oxygen plasma for 60s (150W forward power).

**Step 19**
The wafer surface is primed for photolithographic resist by functionalizing the surface with HMDS vapor, promoting adhesion by replacing the hydrophilic oxidized surface – which attracts a layer of water - with a hydrophobic HMDS surface which is far better for resist adhesion.

**Step 20**
The wafer is coated with SPR220: it is fixed to the spinner by suction, the solution is pooled in the center of the wafer, and the wafer is quickly accelerated to 2000 rpm, at which speed it spins for 30s.
Step 21
The wafer is transferred to a Proximity Oven at 115 deg C for 240s. It then rests on a cooling block for 60s so it can return to room temperature.

Step 22
The mask is exposed onto the wafer via the Karl Suss MA6 contact aligner photolithography system. Due to the relatively low resolution required, the mask is placed into “soft contact” whereby it is held 30 microns from the substrate and there is little-to-no projection magnification. Exposure is from a “flood field”, shooting the whole pattern at once over 3s.

Step 23
The wafer is transferred to a Proximity Oven at 115 deg C for 240s. It then rests on a cooling block for 60s so it can return to room temperature.

Step 24
The wafer is allowed to rest for 15 mins. And think about what it has done.

Step 25
The photolithographic pattern is developed by processing the wafer in the automatic wafer processor, exposing the unmasked portion of the substrate (the pattern).

Step 26
The alignment of the photolithographic pattern (array indices) was verified against the already patterned functional metal layer using optical microscopy (imaging in dark field was particularly effective). If alignment was insufficient, the photolithographic resist was dissolved and reapplied.

Step 27
The pattern exposed in photolithography was etched by subjecting the wafer to Reactive Ion Etching under CF₄ for 10-12 min. This should result in an etched depth of 1-2 microns.

Step 28
Remaining photoresist is striped by bathing the wafer in Shipley 1165 for 30 min.
**Step 29**
The wafer is coated with S1813: it is fixed to the spinner by suction, the solution is pooled in the center of the wafer, and the wafer is quickly accelerated to 4000 rpm, at which speed it spins for 30s.

**Step 30**
The wafer is transferred to a Photoresist hot plate held at 90 deg C, where it sits for 60s. It then rests on a cooling block for 60s so it can return to room temperature.

**Step 31**
The wafer, photoresist face-up, is sealed against a plastic backing and placed into the automated wafer dicer equipped with its all-purpose blade. By rotating the wafer, it is aligned with the dicer’s axes; the corner chip is selected and inter-chip alignment markers are selected in both axes in order to program the spatial increment between chips (and thus where cuts should be made).

**Inspection**
The results of a dose test were inspected via SEM in variable pressure mode, allowing for imaging without the need for an additional conductive layer that would disrupt binding experiments; though preliminary, these results <Figure 33-Figure 36> demonstrated the ability to integrate EM-based inspection into the fabrication process and basic validation for the design of minimal, closely-spaced anchor islands.
Figure 33 - SEM image of anchor pair from preliminary dose test (CtC: 68.1 nm)

Figure 34 - SEM image of anchor pair from preliminary dose test (CtC: 99.1 nm)
Experimental design

As binding is controlled by steric hindrance resulting from the geometry of the host/guest complex (and not the number of receptors available for binding), functionalization should maximize the availability for integrin binding; the PEG solution used will thus be entirely cPEG. Once the anchor sites are functionalized, the substrate will be overlayed by a lipid layer in order to protect the bulk from non-specific binding.70.

Figure 35 - SEM image of anchor pair from preliminary dose test (CtC: 128.1 nm)

Figure 36 - SEM image of anchor pair from preliminary dose test (CtC: 152.1 nm)
In order to develop a calibration curve relating $I_{\text{SERS}}$ to $d_{\text{pair}}$, each chip is saturated with SERS nanoparticles, effectively placing a single scatterer at each anchor site. Each chip thus has an array of $28^2$ distinct pairs devoted to each interparticle separation from 55 nm to 210 nm (centre-to-centre); the pairs can be probed individually or as an ensemble depending on the field of view used in optical sampling.

As per various simulations\textsuperscript{39,40,59,60}, the expected result is an exponential decline from peak scattering (at $d_{ip}$ $\approx$ 2-3 nm) to an asymptotic baseline scattering corresponding to an absence of interaction at a $d_{ip}$ $\approx$ 150 nm ($\approx$ 2.5x the diameter of the particles). The region preceding the peak scattering separation distance, corresponding to the particles in contact and just out of contact, are expected to exhibit an exponential increase from the scattering resulting from the two particles together, where they act approximately like a single asymmetric body and thus exceed the scattering that originates from two independent SERS particles.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{sers_scattering.png}
\caption{Expected relationship between SERS scattering intensity from a pair of SERS particles and the distance separating the particles to be derived from anchor pair platform\textsuperscript{60}}
\end{figure}

From this study, the peak interaction distance, $d_{ip-peak}$, and the interaction distance threshold, $d_{ip-int}$, should be evident to be used and confirmed by studies with the variable density platform.
Density-varying platform: Learning to count

Fabrication Summary

Anchor sites and Indices (photolithography) – layers #1&2
A freshly cleaned wafer was coated first with a lift-off assisting buffer layer (LOR-3A) then a photoresist (S1813), and exposed in a contact photolithography system. The resist is developed, rinsed, and blown dry under Nitrogen. The exposed pattern is etched into the substrate via Reactive Ion Etching under CF4 gas, and the remaining resist is stripped in a bath. The substrate was cleaned via Reactive Ion Etching and then 10nm of Titanium (adhesion layer) and Platinum (functional layer) were evaporated overtop via an electron beam evaporator. Remaining resist was dissolved in a solvent bath, leaving the metalized pattern on substrate.

Packaging
A thick layer of photoresist is spun onto the patterned face of the wafer and baked. The opposite face is sealed against plastic backing, and the wafer is diced into chips.
<table>
<thead>
<tr>
<th>#</th>
<th>Step</th>
<th>Tool</th>
<th>Recipe/Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Pattern mask</td>
<td>Heidelberg</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Clean wafer</td>
<td>e beam spinner</td>
<td>Crossfade from Acetone to IPA</td>
</tr>
<tr>
<td>2</td>
<td>Spin LOR-3A</td>
<td>NBTC spinner</td>
<td>60s @ 4000 rpm</td>
</tr>
<tr>
<td>3</td>
<td>Bake LOR-3A</td>
<td>LOR hot plate</td>
<td>400s @ 190 deg C, Rest for 60s</td>
</tr>
<tr>
<td>4</td>
<td>Spin S1813</td>
<td>Photoresist spinner</td>
<td>30s @ 4000 rpm</td>
</tr>
<tr>
<td>5</td>
<td>Bake S1813</td>
<td>Photoresist hot plate</td>
<td>60s @ 90 deg C, Rest for 60s</td>
</tr>
<tr>
<td>6</td>
<td>Expose pattern</td>
<td>MA6 contact aligner</td>
<td>Soft contact, 30 micron distance, 3s exposure</td>
</tr>
<tr>
<td>7</td>
<td>Develop photoresist</td>
<td>Wafer developer</td>
<td>Soak for 60s in 726 MIF</td>
</tr>
<tr>
<td>8</td>
<td>Rinse/dry substrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Descum</td>
<td>Oxford 80 #1</td>
<td>Etch under Argon for 15s w/75W forward power</td>
</tr>
<tr>
<td>10</td>
<td>Deposit metals</td>
<td>SCCA e beam evaporator</td>
<td>10 nm Ti adhesion layer, 10 nm Pt</td>
</tr>
<tr>
<td>11</td>
<td>Lift off</td>
<td>Container hood</td>
<td>NMP 1165 for 4 hours+, rinse</td>
</tr>
<tr>
<td>12</td>
<td>Spin S1813</td>
<td>Photoresist spinner</td>
<td>30 sec @ 4000 rpm</td>
</tr>
<tr>
<td>13</td>
<td>Bake S1813</td>
<td>Photoresist hot plate</td>
<td>60 sec @ 90 deg C</td>
</tr>
<tr>
<td>14</td>
<td>Dice</td>
<td>Wafer dicer</td>
<td>All-purpose blade</td>
</tr>
</tbody>
</table>

Table 2 - Fabrication procedure of the variable density platform

(see Appendix I: Lithography tools for tool details)
*Fabrication Details*

**CAD:**
Layer #1: Indices (deposited in Ti/Pt)
Layer #2: Variable-density sites (deposited in Ti/Pt)

The 4” wafer was subdivided into a 10 x 10 array of identical ¼” x ¼” chips. Chips featured a 20x20 array of uniform 100 um x 100 um Ti/Pt squares (Layer #1) labeled by two-dimensional indices (01-01 to 20-20) (Layer #2).

Chips were instantiated as a simple, repeating array, each defining an array of binding surfaces and their dynamically parameterized indices.

*Figure 38 - Wafer-level image of CAD describing variable density platform*
Step 0
A standard chrome photolithography mask is patterned by a Heidelberg direct laser pattern writer. A laser writing system, which exposes each mask pixel individually, is chosen over a selective geometric exposure system, such as a PG Mask Writer, given the high total writing area.

Step 1
The wafer is cleaned by spinning it rapidly and spraying it with Acetone then IPA, never allowing it to dry in between.

Step 2
The wafer is coated with LOR-3A: it is fixed to the spinner by suction, the solution is pooled in the center of the wafer, and the wafer is quickly accelerated to 4000 rpm, at which speed it spins for 60s. The LOR, though not photoactive, dissolves much more quickly than a standard photoresist (such as S1813) providing a very clean deposition and liftoff.

**Step 3**
The wafer is transferred to a LOR hot plate held at 190 deg C, where it sits for 400s. It then rests on a cooling block for 60s so it can return to room temperature.

**Step 4**
The wafer is coated with S1813, a positive photoresist: it is fixed to the spinner by suction, the solution is pooled in the center of the wafer, and the wafer is quickly accelerated to 4000 rpm, at which speed it spins for 30s.

**Step 5**
The wafer is transferred to a Photoresist hot plate held at 90 deg C, where it sits for 60s. It then rests on a cooling block for 60s so it can return to room temperature.

**Step 6**
The pattern is exposed onto the wafer via the Karl Suss MA6 contact aligner photolithography system. Due to the relatively low resolution required, the mask is placed into “soft contact” whereby it is held 30 microns from the substrate and there is little-to-no projection magnification. Exposure is from a “flood field”, shooting the whole pattern at once over 3s.

**Step 7**
The photoresist is developed by soaking the wafer in 726 MIF for 60s, leaving the unexposed (masked) portion.

**Step 8**
The wafer is cleaned in the automated rinsing/drying machine.

**Step 9**
Any remaining residue on the wafer surface is removed by Reactive Ion Etching under an Argon plasma for 15s (75W forward power).

**Step 10**
The surface is metalized in an electron beam evaporator under ultra high vacuum. First, a 10nm adhesion layer of Titanium is applied, followed by the 10nm functional layer of Platinum. Platinum evaporation proceeds slowly to avoid any spattering and, once deposited, the substrate must be allowed to cool gradually to avoid any cracking under thermal contraction.

**Step 11**
The remaining (exposed) resist is dissolved by bathing the substrate in NMP1165 for 4+ hours (until metalized layer has visibly “lifted off”), leaving the masked area of the substrate (the pattern) metalized. Rinse with water to fully detach any lingering metal (hardly necessary due to LOR) and blow dry with Nitrogen gas.

**Step 12**
The wafer is coated with S1813: it is fixed to the spinner by suction, the solution is pooled in the center of the wafer, and the wafer is quickly accelerated to 4000 rpm, at which speed it spins for 30s.

**Step 13**
The wafer is transferred to a Photoresist hot plate held at 90 deg C, where it sits for 60s. It then rests on a cooling block for 60s so it can return to room temperature.

**Step 14**
The wafer, photoresist face-up, is sealed against a plastic backing and placed into the automated wafer dicer equipped with its all-purpose blade. By rotating the wafer, it is aligned with the dicer’s axes; the corner chip is selected and inter-chip alignment markers are selected in both axes in order to program the spatial increment between chips (and thus where cuts should be made).

*Inspection*

Once completed, the photolithographic mask was inspected under a light microscope <Figure 41, Figure 42>. It looked great.
Similarly, once wafer was complete, the pattern was inspected under a light microscope <Figure 43- Figure 46>. It looked great.
Figure 43 - Photograph of diced wafer for use in variable-density experiments

Figure 44 – Light microscopy image of diced wafer for use in variable-density experiments focused on intersection between four chips
Platform usage

In order to achieve a varying density of integrin binding sites across chips, the ratio of mPEG to cPEG in the PEG solution was varied from one absolute to the other. By increasing the concentration of cPEG randomly bound to the substrate, more integrin receptors could ultimately be anchored, increasing the number of binding sites for SERS nanoparticles. Assuming that sites can be saturated with bound particles, this strategy acts as a proxy for the systematic increase in the density of particles bound stochastically on the planar surface. Probing $I_{SERS}$ across the surface thus offers an aggregate assessment of scattering intensity as a function of particle density (in inverse proportion to mean distance interparticle distance, $d_{tp}$).
Using the results from the first platform, $I_{\text{SERS}}(d_{ip})$, it is expected that measuring SERS intensity as a function of Integrin density will manifest four regimes of scattering behaviour:

i) Unbound: given typical binding behaviour, it is expected that stable binding might require a plurality of available receptors; it is thus expected that, at the lower end of receptor binding, no signal will be detectable, even if single particles are provide sufficient signal intensity

ii) Non-interacting: as the density of integrin receptors rises, stable binding occurs with an average interparticle distance greater than interaction distance threshold; scattering intensity will thus rise linearly with integrin concentration

iii) Interacting: as the density of integrin rises, the mean distance between new binding sites is less than the interaction threshold; scattering intensity now rises exponentially with integrin concentration

iv) Fully saturated: eventually, whether due to steric interference, binding dynamics, or transport dynamics, additional integrin receptors bind with decreasing effectiveness and the increase in net scattering slows to an asymptote

![Figure 47 - SERS scattering intensity with increasing density of receptors](image)

The point of inflection transitioning between the second and third regimes corresponds to the point where the $d_{ip} = d_{ip\text{-int}}$.

**Discussion**

**Multimodal data correlation**

From a technology standpoint, automated imaging over either a given array (in the case of anchor pairs at given separation distance) or an entire anchor site (in the case of the variable density platform) and
aggregating the results into a single metaimage were a simple challenge – automation protocols and stitching algorithms were *pro forma* in both the SEM and confocal Raman systems used in demonstration. Given the regular, simple, and well-indexed overall structures, intermodal coordination/overlay of the metaimages would be relatively trivial. The challenge would come from leveraging the mapping information from SEM imaging to inform expectations regarding scattering behaviour.

In the case of anchor pairs, this could be as simple as verifying the presence of a single nanoparticle at each site and the effective distance between them, an independent method of verifying the underlying assumptions that all sites are occupied and that each binds only a single particle; though an expected signal range for $I_{\text{SERS}}$ could enforce a single one of those conditions, the conflation of the two would require a second method – dark field imaging might also have sufficed with sufficient colour (ie plasmon peak) sensitivity. The former verification could have provided a test for the rejection of data from failed binding sites whereas the latter would have provided a precise measurement for interparticle separation distance, mitigating any inherent variation in fabrication and binding.

For the variable density platform, the challenge would have been to map the particles then use the relationship between interparticle distance and scattering intensity derived from the first platform to inform modeling of the scattering originating from high particle density. Image recognition algorithms would have made short work of mapping particle positions but significant advances would have had to have been made in terms of multibody effects in Plasmon coupling.

**Predictability of probe**

Given the sensitivity of the relationships being studied, the predictability of probes represents a critical source of error. Fluctuation in Raman scattering intensity originating from a probe represents a significant conflating factor in both deriving $I_{\text{SERS}}(d_{ip})$ and applying it to variable density regions. Given the electrostatic means of binding Raman reporters onto the SERS label, there would seem significant opportunity for variation on the individual particle level. Though perhaps sufficiently mitigated in the statistical number of sampling data points, this might lead toward a more predictable label construction\textsuperscript{71}.

**Determining threshold density of utility. And beyond.**
Outside of establishing an experimental demonstration of $I_{\text{SERS}}(d_{ip})$, the first goal would be to establish the density bounds for the various regimes of receptor binding, starting with the trivial regime wherein direct linear interpolation is possible. Whereas the upper bound will be directly implied by the point where the rate at which $I_{\text{SERS}}$ begins to increase exponentially with integrin concentration, it will be substantiated by $d_{ip,\text{intr}}$, the distance of separation between two particles whereby they cease to interact. By knowing the area of the field of view and $d_{ip,\text{intr}}$, the number of non-interacting particles that can be fit can be interpolated, giving a theoretical maximum density at which particles can still scatter independently.

Beyond this linear regime is where the fun starts. When the signal exceeds this threshold, modeling will use $I_{\text{SERS}}(d_{ip})$ to extrapolate from the measured $I_{\text{SERS}}$ and estimate the number of bound particles that are generating the signal.

**Justifying diagnostic thresholds**
In the ultimate application of SERS labels as clinical diagnostic tools, a critical requirement will be a signal threshold for a label, beyond which a corresponding receptor can be understood as present on a cell. This threshold, in practice represented as a “normal” clinical range, will be determined through a statistical sampling of patient cells diagnosed through other means but knowledge of the SERS intensity resulting from a high density of labels could give *a priori* knowledge of this range based on our existing understanding of cell biology. Moreover, the process could work in reverse: SERS measurements could inform understanding of receptor density, position, and movement in binding. This could be especially useful in calibrating a clinical process to a new set of labels. Given an understanding of the theoretical scattering behavior of SERS particles bound in the relevant configuration, knowledge of the independent Raman character of the particles would allow for the advance prediction of those clinical standards.

**Binding dynamics studies**
Another potential application for the platform would be studying the binding kinetics of receptor systems. Biological systems could be simulated through the patterning of anchor sites on the substrate, and the systematic saturation of the bound receptors with SERS nanoparticles at various concentrations. Binding dynamics could be understood by monitoring $I_{\text{SERS}}$ over time, relating the bound concentration of particles with the concentration applied.
Conclusion

In the experiments undertaken and planned, significant progress was made toward the goal of improving cancer diagnosis. Work toward developing immunotargeted labels differentiable under dark field microscopy demonstrated not only a basic ability to target surface receptors characteristic of cancerous cells but the ability to co-image nanoparticles with cells and differentiate between alloy nanoparticles based on their constitution. Efforts to study the quantitative analysis of SERS NPs within a diffraction-limited probing area via their SERS signal evolved a strategy for study, a design layout, and a fabrication strategy for the requisite experimental platforms.

Dark field cell surface receptor labels
Sample preparation, mounting, and imaging techniques were refined for the imaging of 10-60 nm particles and fixed patient cells under dark field light microscopy using traditional optics, light source, and imaging electronics. CD45 receptor-targeting on patient leukocytes using commercial 60 nm gold particles was demonstrated in dark field imaging and confirmed with flow cytometry studies. The differentiability of alloy nanoparticles (≈30 nm) under dark field light microscopy was demonstrated and preliminary CD45 receptor-targeting on patient leukocytes was demonstrated through imaging.

Quantifying high-density surface-bound SERS particles
Two experimental platforms were designed to facilitate the interpretation of the SERS signal originating from a dense region of surface bound particles. The first platform, arraying anchor site pairs at set distances from each other for binding by SERS particles, would offer an experimental relationship between the distance separating a pair of particles and their sum scattering intensity. The second platform, consisting of an array of large binding surfaces functionalized with densities of binding sites increasing to saturation, would experimentally establish the relationship between the binding density and the intensity of the SERS signal originating from stochastically surface-bound particles over a range of densities. Fabrication processes were designed for both platforms; the former was tested and the latter was completed.
Appendix I: Lithography tools

Software
L-Edit CAD Software
http://www.tannereda.com/l-edit-pro
CAD software for pattern design, offering multi-layer and multi-material definition, arrayed instantiation, scripting, and exporting to GDSII format for conversion into machine-specific instructions.

GenISys Layout BEAMER - E-beam Pattern Conversion and Proximity Correction
Process flow directed software that imports GDSII layout files, extracts and converts cells and layers, corrects for proximity effects, and outputs machine-specific instructions for lithography systems.

Tools
FilMetrics Film Measurement System F50-EXR
http://www.filmetrics.com/thicknessmeasurement/f50
Given a model of the refractive index (n) and dispersion (k) of optically transparent films layered on a known substrate, the thickness of films can be measured using a photospectrometer.

CVC SC4500 Combination Thermal/ E-gun Evaporation System
Cryopumped combination thermal and electron beam evaporation system that allows for 4 materials evaporated by a 10 kW electron beam and 3 materials via a 3kW thermal source. Selectable material source allows for sequential evaporations, layering materials without breaking vacuum.

JEOL JBX-9300FS Electron Beam Lithography System (100kV)
High resolution electron beam patterning with a resolution < 20 nm over a 9” write area with a field size 1mm (stitching accuracy of ±25 nm).

Oxford PlasmaLab 80+ RIE System
RIE system dedicated to anisotropically etching silicon dioxide, silicon nitride, and silicon substrates, and surface processing with CHF3, CF4, SF6, argon and oxygen gases. The 500 W 13.56 MHz RF power source operates under turbo-pumped pressures from 10 - 1000 mT.

Zeiss Supra SEM (100V-30kV) with high and variable pressure modes
High resolution Scanning Electron Microscope offering resolutions as low as 1.0 nm at 15 kV and 1.7 nm at 1 kV at High Vacuum mode, and 2nm at 30kV at Variable Pressure mode. Both offer secondary electron detectors.

Heidelberg Mask Writer DWL2000 Laser Pattern Generator and Direct Writer
Laser writer that patterns the mask pixel-by-pixel, ideally suited for high resolution writing and for masks with high patterned area. Using the 4mm write lens, there is a nominal resolution of 0.7um and writing rate of 110mm²/min.

Hamatech-Steag HMP900 Mask processing system
Automated mask processing system that develops the patterned resist and etches the chrome mask layer accordingly.
Nikon Eclipse L200N microscope and Nikon Elements D image capture software (bright and dark field condensers)
Standard light microscope with 40x objective and image capture software.

YES LP-III Vapor Prime Oven (HMDS)
A vacuum oven that primes wafers for the adhesion of photolithographic resist with HMDS vapor. Clean wafers are dehydrated by cycling between 150 deg C evacuation and filling with dry nitrogen. The evacuated chamber is then filled with HMDS vapor, forming a monolayer on the wafer surface.

Karl Suss MA/BA 6 Contact Aligner (280-350 nm lamp)
Large wafer-compatible (up to 150mm) contact photolithography system that flood exposes the masked pattern onto the wafer. Infrared imaging allows for backside alignment and an overlay accuracy of 1 micron. "Contact lithography", where the mask is in close contact with the wafer, is available at various nominal distances; for applications where mask resolution is sufficient, this allows for simple alignment and no wear on the wafer.

Oxford Plasmalab100 RIE System (fluorine-based ICP deep SiO2 etching)
An inductively coupled plasma based system for deep SiO2 etching; compatible with CHF\textsubscript{3}, CF\textsubscript{4}, C\textsubscript{2}F\textsubscript{6}, C\textsubscript{4}F\textsubscript{8}.

K&S 7100 Dicing Saw
Automated wafer dicing saw that, upon initial alignment and specifying chip spacing, cleanly divides a wafer into constituent chips.

Solutions and Materials
Fused silica wafer (100mm)
Microposit S1813 Photoresist (+)
Megaposit SPR220 Photoresist (+)
LOR-3A: Lift-off Resist
AZ 726 MIF Developer
Shipley 1165 NMP Remover
Aluminum etch (Phosphoric Acid 40-80%, Acetic Acid 3-20%, Nitric Acid 1-5%)
495K PMMA (2% in Anisole) - Electron beam resist (+)
950K PMMA (2% in MIBK) - Electron beam resist (+)
MIBK:IPA (1:3) - PMMA developer
Methylene Chloride – PMMA dissolver
References


