Quantitative Analysis of the Tumor Immune Burden Using Targeted Nanoprobes

James J. Slade Honors Thesis

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Abstract—Current methods for immune therapy monitoring and screening involve the use of invasive biopsies that cannot provide real-time information. To address this limitation, our goal is to use rare earth albumin nanocomposites (ReANCs) to develop a non-invasive, real-time monitoring and screening tool for immunotherapy response. ReANCs emit signal in the shortwave infrared region (SWIR) of the spectrum when excited by near infrared (NIR) radiation, allowing them to be used for optical imaging of biological tissue. Given the variety of functional groups and drug-binding pockets on their albumin shells, they can also be functionalized with different targeting ligands for precision targeting. In this project we present the initial steps in devising a method to monitor immune burden around tumor spheroids in vitro using differentially calibrated ReANCs. ReANCs were functionalized with folic acid using EDC, a zero-length crosslinker, for precision targeting of tumor cells. ReANCs will be functionalized with an anti-CD8 antibody fragment to target the CD8 receptors present on cytotoxic T cells. 4T1 murine breast cancer cell spheroids containing varying concentrations of albumin nanocomposites (ANCs) were grown and imaged. Quantitative methods were employed to develop an algorithm to classify spheroids based on relative particle loading by analyzing fluorescent signal from Janelia Fluor 646, a fluorescent dye encapsulated in the ANCs, but data showed limited correlation between pixel intensity of spheroid images and relative loading percentage. Adjustments in the spheroid formation protocol will be made to address the variability and limited correlation in the spheroid data. Work done on this project will serve as the foundation for future work in in vitro tumor immune microenvironment monitoring and ultimately in in vivo studies.

I. INTRODUCTION

Immunotherapy has emerged as a major modality for cancer treatment. Checkpoint inhibitor drugs have been approved in certain cancer types such as melanoma, non-small-cell lung cancer, and renal cell carcinoma and have shown promising results [1]. However, the subset of the population that responds to immune therapy is still fairly low and response is highly variable between patients [2]. Since immunotherapy can be both a physical and economic burden to the patient, identifying individuals who will be most responsive to treatment is important.

It has been shown that the number and type of immune cells surrounding tumor sites plays a role in determining patient response to immunotherapy [3]. Current methods for immune therapy monitoring utilize this characteristic of the tumor microenvironment to determine the progress of immunotherapy treatment. Tissue samples are analyzed for the presence of specific biomarkers indicative of treatment progression [4]. These methods, since they require the use of invasive biopsies or blood samples, cannot provide real-time information about the tumor microenvironment [5]. Therefore, there is a clear need for a non-invasive, real-time monitoring and screening tool for immunotherapy response.

The goal of our project is to work towards developing a non-invasive, real-time monitoring tool that would allow for simultaneous imaging of tumor sites and the surrounding immune cells in order to quantifiably assess and screen for immune therapy progression. We will do so by employing the use of rare earth albumin nanocomposites (ReANCs).

ReANCs consist of a rare-earth particle core and an albumin protein shell. Rare-earth particles emit light in the shortwave infrared (SWIR) region (wavelength=1000-1700 nm) of the electromagnetic spectrum when excited with near infrared (NIR) radiation (wavelength=980 nm). The SWIR region of the spectrum offers a significant advantage in the context of biological imaging because there is little tissue autofluorescence in this region. This allows for deeper penetration and less scattering [6]. The specific wavelength of light emitted by these particles is tunable and is based on the specific rare-earth composition being used. Because of this, distinct populations of rare-earth particles can be used simultaneously to image distinct subpopulations of cells [7].

We have shown the ability of these particles to image tumor microlesions in a mouse model of human breast cancer and display real-time cell activity [8]. These particles, given the variety of functional groups and availability of drug-binding pockets on their albumin protein shells, can also be functionalized with targeting ligands for precision targeting of distinct cell populations [9].

In this project, we will leverage the unique biochemical
and optical properties of ReANCs to work towards the first steps in developing a monitoring and screening tool for cancer immunotherapy. We will functionalize distinct subpopulations of ReANCs with folic acid and a CD3-blocking peptide to target both tumor cells and the surrounding t-cells. Doing so will allow us to simultaneously image tumor sites and the surrounding immune burden. We will also develop a quantitative immune index using loading of albumin nanocomposites (ANC) fluorescently labeled with Janelia Fluor 646 in a tumor cell spheroid model. By utilizing a cell spheroid model, we will be able to mimic the 3D optical environment in an in-vivo setting. These two goals will serve as the foundation for future work in creating the non-invasive, real-time monitoring tool.

Figure 1 above briefly illustrates how differently configured ReANCs will ultimately be used for future in-vivo real-time monitoring.

II. Equipment and Methodologies

A. ReANC Synthesis

The particles were created via controlled coacervation. Human serum albumin (HSA) was prepared at a concentration of 20 g/mL in 10 mM NaCl solution at pH 8.5. Erbium rare earth particles and Janelia Fluor 646 were dissolved in ethanol and infused into the albumin solution at a rate of 1.5 mL/min. The HSA formed a shell around the rare earth and fluorophore core. Glutaraldehyde was then added as a cross-linking agent. The addition of Janelia Fluor 646 was helpful for subsequent confocal microscopy, as it emits signal in the visible light region of the spectrum upon excitation (Ex: 655 nm, Em: 671 nm), allowing for easy visualization and localization of the particles. Albumin nanocomposites (ANCs) were also created for this study, particularly for the immune indexing experiment. The method for the creation of these particles was identical to that of ReANCs besides the addition of rare earths in the ethanol solution.

B. ReANC Functionalization

Folic Acid Functionalization: Free amine groups on the ReANCs can be conjugated to molecules with carboxyl groups with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) as a zero-length cross-linker. This method was used to conjugate folic acid onto ReANCs.

CD3 Functionalization: Similarly to the folic acid functionalization, EDC will be used as a zero-length cross-linker to bind the peptide onto the cells.

C. Tumor Cell Spheroid Generation

4T1 murine breast cancer cells were plated on a 96-well flat bottom plate and were either left untreated or treated with ANCs containing Janelia Fluor 646 as a fluorophore. ANCs, rather than ReANCs, were used because they are more cost-effective and simpler to make. Treated and untreated cells were combined in varying proportions to create solutions consisting of 0%, 25%, 50%, and 100% prelabeled cells by volume. 300 cells were plated on Nunclon U-Bottom Sphera plates to create individual spheroids. The spheroids were left to incubate for 3 days to allow for proper spheroid formation.

D. Tumor Cell Spheroid Imaging

Tumor cell spheroids were transferred to flat bottom plates for imaging. The spheroids were imaged using a Zeiss LSM 780 confocal microscope. Z-stack images capturing brightfield and Janelia 646 signal were taken of the spheroids.

E. Tumor Cell Spheroid Image Processing and Analysis

Image Processing: Z-stack spheroid images were processed using ImageJ. Fluorescence signal intensity of individual image slices was recorded and stored for subsequent analysis. Binary masks were drawn manually and saved for each spheroid for future use as well.

Quantitative Analysis: Fluorescence signal intensity and particle loading were measured and plotted.

Machine Learning-Based Analysis: Maximum intensity of spheroid image stacks were computed and saved for both the brightfield and fluorescence channels. Masked composite images were created with brightfield and fluorescence signals in different channels. Random Forest classification was used as a learning method to classify spheroid images by loading percentage using the fluorescence image data. The algorithm was implemented using the sci-kit learn library in Python. A deep learning approach using convolutional neural networks (CNNs) for image classification was implemented using Tensorflow and Keras, a Python library that allows for rapid prototyping of neural networks and associated functions.

III. Results and Discussion

A. ReANC Synthesis

A standardized protocol for ReANC synthesis has been developed and properties of the resulting ReANCs have been well-documented previously in the lab [9]. ANCs with Janelia-Fluor 646 as a fluorophore were created and used for spheroid prelabeling.

B. ReANC Functionalization

Many lines of cancer cells show an over-expression of folate receptors on their surface [10]. Therefore, we chose to use folic acid as a targeting ligand for precision targeting of tumor cells. The folic acid functionalization protocol has been developed and standardized in the lab. Increased cellular
uptake with folic acid functionalized ReANCs compared to non-functionalized ReANCs was shown using flow cytometry (FACS). Flow cytometry results are shown in Figure 2 below.

As shown in the figure, there was a significant increase in uptake of functionalized ReANCs as compared to non-functionalized ReANCs based on mean-intensity (p=0.0032). We also confirmed that the folic acid functionalized ReANCs were binding to folate receptors specifically on the tumor cells by conducting competitive inhibition studies. We saw a decrease in uptake of folic acid-functionalized ReANCs by tumor cells when free folic acid was present in solution, confirming that our particles were targeting the folate receptor specifically. The results from our functionalization targeting validation experiments confirm the potential use of folic acid as a targeting ligand for precision targeting of tumor cells moving forward. Functionalization of ReANCs with a CD3-blocking peptide is ongoing. Similar assays for cellular uptake and target specificity will be done once the protocol has been optimized.

C. Tumor Cell Spheroid Imaging

A total of 184 spheroids over 8 experiments were grown and imaged. Table 1 below shows a breakdown of the spheroids based on relative loading percentage.

<table>
<thead>
<tr>
<th>Loading</th>
<th>0%</th>
<th>25%</th>
<th>50%</th>
<th>75%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td># Spheroids</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>36</td>
<td>37</td>
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</tbody>
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Figure 3 shows representative images showing cross-sections of z-stack images of spheroids of cells with varying concentrations of ANC loading. The images contain the brightfield signal (grayscale) overlayed with the fluorescence signal (red) from the Janelia-Fluor 646 fluorophore.

D. Analysis of Spheroid Data

Spheroid pixel intensity data was analyzed to measure its ability to classify spheroid images based on percentage of prelabeled cells. Raw integrated pixel density, which refers to the summation of the pixels in an image selection, was measured for the Janelia-Fluor 646 channel within regions of interest containing the actual spheroids. Pixel intensity data for the individual spheroids is plotted below using three different metrics in order to visualize any correlation between pixel intensity and percentage of prelabeled cells.

Figure 4 shows three box plots comparing pixel intensity of spheroids based on relative ANC loading using three distinct metrics: average intensity (top left), maximum intensity (top right), and control-normalized average intensity (bottom center). For all metrics, pixel intensity was normalized to the cross-sectional area of the spheroids as manually drawn in ImageJ in order to take into account variations in spheroid size. For the average intensity metric, normalized pixel intensity was computed for each slice in the z-stack image for a spheroid and these values were then averaged to get a final average intensity value for that spheroid. Similarly, the maximum value from the individual normalized pixel intensity values for the z-stack image was taken to get a final maximum intensity value for a spheroid. The control-normalized average value metric was computed by considering each experiment separately. Within an experiment, the average value of the average intensity metric for all of the control (0% loaded) spheroids was computed. This value was then subtracted from all of the average intensity values for the spheroids of that specific experiment. As shown in the above figure, there was no significant correlation between pixel intensity of the spheroids and percentage of prelabeled cells. The only metric that seemed to show any trend along the median values was the control-normalized average intensity metric (bottom center), but this was weak and could not be used to classify and index spheroids.

The utility of machine learning methods for spheroid clas-
classification was limited due to the small dataset size. However, these methods were still explored for their potential to illustrate trends in the data and to develop analysis pipelines for future use. Maximum intensity projections, in which the maximum pixel values are selected along the z-axis of a stack of 2-dimensional images to create one 2-dimensional projection, were created for each of the spheroids in order to create singular images that captured important features of the original z-stack images varying in stack size. The maximum projection would highlight the presence of individual fluorescence signal points, and so was considered suitable for this application in which total fluorescence signal was important. Multiple machine learning algorithms were tested for their potential application including random forests. This model was utilized because of its applicability to image classification problems and its ability to prevent overfitting. The algorithm was implemented using the sci-kit learn module available in Python. The model was trained using 80% of the total data with 20% of the data left for testing. The prediction accuracy using this model was 0.24 on testing data, only slightly better than random choice.

Convolutional neural networks are useful in image classification because they are able to extract important spacial information from the inputs. Such networks typically rely on large amounts of data in order create a useful classifier without overfitting of the training data. However, these networks can be adapted to fit smaller datasets using a method called transfer learning. Pre-trained neural networks trained to classify certain images can be adapted and slightly changed to accommodate a different set of images. Convolutional layers of neural networks extract features from images gradually. Image features applicable to most images, such as edges and curves, are extracted early on in the network, which can prove valuable for most image classification tasks. Transfer learning involves utilizing a pre-trained network and only retraining later layers while freezing earlier layers so that basic feature extraction can be done before more domain-specific information is extracted for classification. The applicability of transfer learning for fluorescence image classification on limited amounts of data has been shown to provide good results, which suggested that the prototyping of such methods on our spheroid images could prove to be useful. The maximum projection of the brightfield signal, the maximum projection of the fluorescence signal, and the manually drawn binary mask were scaled and combined to create single RGB images that would be used to get a single composite spheroid image that captured all of the image data. A representative set of individual images and the corresponding composite image is shown in Figure 5 below.

Fig. 5. Composite Maximum Spheroid Projection

Brightfield Fluorescence Mask Composite

DenseNet121, a common convolutional neural network structure pre-trained on ImageNet, was used as the base model for transfer learning testing, as this network was shown to provide good results for fluorescence image classification. The convolutional layers of this network were frozen and a new classifier was added to the top of the network. Training this model, with 80% of the data for training and 20% for validation, yielded a validation accuracy of 0.36. This accuracy, like with the other machine learning models tested, was not of any predictive value.

The limited ability of our machine learning models to accurately classify the spheroid images could be attributed to issues in image representation, processing and hyper-parameter optimization. However, the fact that the predictive accuracy was very close to that of random chance (0.2) suggests that the actual training data used could have been suboptimal. The pixel intensity data further supports this claim. Differences in pixel intensity of spheroids of different percentage of pre-labeled cells was not significant enough to suggest that there was any correlation between the two. Since pixel intensity in the fluorescence channel is the primary feature of nanoparticle presence, this suggests that the differences in the concentration of nanoparticles was not clearly visible in the spheroid images.

The spheroid growth and plating protocol for this project involved plating three hundred cells/well and allowing the spheroids to grow and form over the course of two to three days. By doing so, it is possible that the growth of additional cells during the incubation period could have diluted the fluorescence signal to a point where differences could not be detected. Furthermore, we also found that dead cells created necrotic cores at the centers of the spheroids, which has been shown to, and did, emit significant fluorescent signal. This was also most likely the reason why some of the control (0% prelabeled) spheroids had non-zero pixel-intensity readings.

Moving forward, we will use a different protocol for growing the spheroids to see if doing so limits these confounding effects on the particle fluorescence signal. We will try plating more cells and will keep them to incubate for less time, enough to allow them to adequately form coherent spheroids. By doing so, we hypothesize that additional cell growth will be lessened and that necrotic cores will not form as significantly in the spheroids.

IV. Conclusion and Future Directions

In this project, we have begun the first steps towards ultimately creating a multi-spectral imaging tool for immune therapy monitoring. We have fabricated folic-acid functionalized ReANCs and have shown that these particles can be leveraged for precision targeting of tumor cells. We are currently optimizing a CD3-blocking peptide functionalization protocol as well.

We have also worked on developing a ratiometric index to determine ANC loading of 4T1 cell spheroids. Quantitative analysis of the pixel intensity of spheroid z-stack images showed limited correlation between percentage of pre-labeled cells and pixel intensity. Machine learning-based methods
were also explored, but a similar lack of correlation was found. We will address these shortcomings of our data moving forward by working to create a new protocol for growing and plating the cancer cell spheroids in order to create cleaner, more informative images. We will adapt the same analysis methods as employed on our current spheroid images and, where applicable, work on optimizing such methods with the better image data.

Once the functionalization protocols have been optimized and the ratiometric index has been developed, we will begin working on monitoring immune burden around tumor cell spheroids. This will be done using µ-Slide 1 Luer from Ibidi, placing a 4T1 tumor cell spheroid in one well, immune cells in the other, and monitoring, in real time, how the immune cells interact with the spheroid. We will then utilize our existing algorithm or devise a similar one to create a predictive index of immune burden. A representative figure of the setup is shown below.

Fig. 6. Immune Cell Migration Set Up

REFERENCES


