Assessing Quality of Tissue Engineered Retinal Pigment Epithelia Using Absorbance Imaging & Artificial Intelligence

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Carl Simon
Age-Related Macular Degeneration & Retinal Pigment Epithelial Cells (AMD & RPE)

- RPE support rods & cones by delivering nutrients from the bloodstream & removing waste that the rods and cones generate
- In AMD, RPE stop performing their support functions & rods & cones die, resulting in loss of central vision
- AMD is a common cause of vision loss in developed countries, affecting 30 to 50 million people worldwide
  - No good treatment for 90% of AMD cases (dry AMD)
- **Treatment Goal:** Manufacture healthy RPE & deliver to eye to prevent rod and cone cell death

Formation of tight junctions is key for RPE function

RPE Manufacturing in Bharti Lab at NIH: Takes 155 Days Per Patient

- Draw patient blood, centrifuge, retain PBMC fraction
- Isolate CD34+ monocytes using magnetic beads
- Generate bank of clinical grade induced-pluripotent stem cells (iPSCs) using 4 episomal factors

**Blood** → **CD34+ Peripheral Blood Monocytes** → **iPSCs** → **Neuro-ectoderm** → **iRPE progenitors** → **Committed iRPE** → **Immature iRPE** → **Mature iRPE**

![Image of iPSC-derived RPE (iRPE)](image)
Pigmentation Correlates with RPE Maturation

- RPE express melanin to absorb light
- Prevents light from entering the back of the eye
  - Reduce light scattering in the eye to improve vision
  - Protect tissue from exposure to light, reduce disease & cancerous lesions
- NOTE: Pigmentation is an indirect measure of function
  - *Pigmentation is not part of the mechanism of action for treating AMD*
  - MOA = support of the rods & cones

Cruz et al. 2018: Phase 1 Clinical Study of an Embryonic Stem Cell-Derived RPE Patch in AMD

“Appearance” is a key quality attribute

**VIR = Visual Inspection Release test**

- **Appearance and viability**
- **Karyotype**

- **Appearance and viability**
- **Sterility and mycoplasma**

- **Appearance and viability**
- **Sterility**
- **Cell count**

- **Appearance and viability**
- **Mycoplasma**
- **Lin28 ISH (impurity)**
- **PMEL17 ICC (purity)**

- **VIR test**
- **Sterility, endotoxin, and mycoplasma**

**“RPE cells were assessed using a light microscope for pigmentation, cobblestone morphology, health and signs of contamination and processed further, only if they passed this visual check.”**

**“On the day of surgery...The patch is assessed visually through the clear lid of the storage container for integrity, pigmented cell coverage and viability.”**
Measurement Issue

Pigmentation Correlates with RPE Maturation

How to replace “Appearance” qualitative visual inspection tests using image as a key quality attribute of iRPE patches with quantitative measurements?
Quantitative Bright-Field Absorbance Microscopy (QBAM)

- Use brightfield microscope as a spectrophotometer
- Each pixel in an image is a *quantitative* measure of pigmentation

Calculate Absorbance Image

\[ A = -\log_{10} \frac{I_{\text{Cell}} - I_{\text{Min}}}{I_{\text{Max}} - I_{\text{Min}}} \]
Experimental Overview

The information in the pixels is used to assess RPE quality by 2 approaches:

1. Deep neural network (DNN): uses image-level spatial patterning of pixel intensities
   • Avoids image processing/feature engineering
   • Creates a “Black box” model
   • Tissue-level & cell-level

2. Traditional Machine Learning (TML): uses single cell-level metrics: shape, intensity, texture
   • Requires extensive image processing/feature engineering
   • Provides some biological insights based on important cell metrics/features
   • Cell-level only
Data Collection Routines:

- Data collection automated using custom plugin for MicroManager
- Starts with “microscope stability” routine where it collects images at different exposure times until 95% confidence interval of each pixel is 0.01 absorbance units
- The best exposure time for each pixel is used to generate an “optimized” image
- Takes images at 3 wavelengths by using three different filters (red, green, blue)
  - This helps to account for scattering, since scattering is less dependent on wavelength than is absorbance
Assessing comparability using QBAM to measure absorbance of 3 different neutral density filters imaged on 3 different microscopes using different filters.

Stability metric = $-\log_{10}\left(\frac{I_1}{I_2}\right)$

- Capture 3 images and average ($I_1$)
- Capture another three images and average ($I_2$)
- The microscope is stable if stability metric is $0 \pm 0.0001$
Experimental Overview

1. Patient Blood
2. iPSCs
3. Immature iRPE
4. Seed into Transwell Plates
5. Culture 8 weeks with weekly measurements of: QBAM, TER & VEGF
6. Convert to Absorbance Images
7. Segment cell borders in QBAM by DNN-S
8. Determine single cell metrics using WIPP
9. Train DNN-F to predict TER & VEGF from QBAM using 5/6 of the data
10. Test DNN-F on 1/6 of the data
11. Train TMLs to predict TER & VEGF from WIPP cell metrics using 5/6 plates
12. Test TML on 1/6 of the data
Experimental Overview

**Goal for DNN-Z & DNN-S:** Segment single cells in QBAM images
- By using QBAM images to predict where a human would identify a cell border in ZO-1 immunostained fluorescence image
**Experimental Design**

Train on 5 plates…test on 1 plate

- 12-well transwell plates
- 6 plates x 6 wells/plate = 36 wells
- iPSC-derived RPE from healthy donor
- 3 treatments (control, aphidicolin, HPI4)
- QBAM
  - 12 overlapping fields of view per well (4 x 3) per time point
  - Use three different color filters (red, green, blue)
- TER measurement on all wells and plates 1X/week for 6 weeks
- VEGF measured on each well in 2 plates 1X/week

<table>
<thead>
<tr>
<th>Week</th>
<th>QBAM</th>
<th>TER</th>
<th>VEGF Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>X</td>
<td>X</td>
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</tr>
<tr>
<td>4</td>
<td>X</td>
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<tr>
<td>5</td>
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<tr>
<td>6</td>
<td>X</td>
<td>X</td>
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</tr>
<tr>
<td>7</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>8</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
Generating Good & Bad RPE: Aphidicolin & HPI4

Aphidicolin
• **inducer** of RPE maturation
  • antibiotic that inhibits eukaryotic nuclear DNA replication and blocks the cell cycle at early S phase

HPI4
• **inhibitor** of RPE maturation
  • hedgehog pathway inhibitor-4, HPI4
  • Hedgehog signaling pathway transmits information to embryonic cells required for proper cell differentiation
RPE Functional Attributes:

1) Trans-Epithelial Resistance (TER) &
2) Polarized VEGF Secretion (VEGF Ratio)

Tight junctions are key for RPE function

Tight junctions: multiprotein junctional complexes common in epithelial cell layers that function to prevent passage of solutes & water between cells

Fun Facts

Scope

- iRPE Manufacturing takes 155 days
  - Bharti lab has 2 dedicated staff that only manufacture RPE for use by the rest of the lab
  - $10K in growth factors required to make a batch of RPE
- Cost per patient is unknown (~$1M per patient)
- iPSC technology is new, discovered in 2006, only 6 patients have rec’d them
  - 6 in Japan (1 iRPE patch & 5 iRPE suspensions)
  - 0 in USA
- Data from iRPE from 10 donors
- Implemented 53 DNN & ML AI routines to
- Data Dissemination (600 GB): https://isg.nist.gov/deepzoomweb/data/RPEimplants

Challenges

- Reliability: microscope stability, optimal exposure time, background, blank, 3 colors, different wells
- Speed: Optimizing speed to minimize time that cultures are out of the incubator
- Big data: 200K images, 1TB, 12M single cells
  - 4 channels of data: QBAM RGB + ZO-1 fluorescence
  - organizing, moving, annotating
- Processing: background subtraction, generating absorbance images from brightfield images
- Stitching: 4x3 tiling from each well
- 7 summer students participated in hand segmentations
- Build prototype at NIST & convince NIH to install it
- GLP: NIH gave us access to GLP facility (risky for them)
Quantitative Brightfield Absorbance Microscopy (QBAM)

**Week 1**

- **HPI4** (inhibits differentiation)
- **Control**
- **Aphidicolin** (induces differentiation)

**Week 8**

- **HPI4** (inhibits differentiation)
- **Control**
- **Aphidicolin** (induces differentiation)
Each data point is a well

Weekly QBAM imaging did not impact iRPE maturation
**Mean Absorbance vs. AI: Trans-Epithelial Resistance (TER)**

Quality threshold set at “400” (could be refined after clinical trial)

Root Mean Squared Error (RMSE): 70.6 $\Omega \cdot \text{cm}^2$

Accuracy: 94%, Sensitivity: 1.0, Specificity: 0.9
Quality threshold set at "3.0" (could be refined after clinical trial)

Root Mean Squared Error (RMSE): 1.1
Accuracy: 100%, Sensitivity: 1.0, Specificity: 1.0

Mean Absorbance vs. AI: VEGF Ratio
Deep Neural Network Prediction of RPE Image Segmentation

Comparative histograms of 44 cell image features of RPE segmented by hand or by DNN-S:

- $F_2 = 0.71$
“Cobblestone Network”

Number of Neighbors

Avg. Num. Neighbors

Week
Machine Learning Prediction of TER from WIPP Single Cell Features

**Summary of Algorithm Regression Errors**

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>TER Root Mean Squared Error ($\Omega \cdot \text{cm}^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNN-F: Deep Learning Neural Network</td>
<td>70.6</td>
</tr>
<tr>
<td>MLP: Multi-Layer Perceptron</td>
<td>84.7</td>
</tr>
<tr>
<td>PLSR: Principle Least-squares Regression</td>
<td>100.1</td>
</tr>
<tr>
<td>L-SVM: Linear Support Vector Machine</td>
<td>102.7</td>
</tr>
<tr>
<td>RR: Ridge Regression</td>
<td>109.6</td>
</tr>
<tr>
<td>RF: Random Forest</td>
<td>116.4</td>
</tr>
</tbody>
</table>

**Figure Notes:**
- Dashed line represents a perfect prediction.
- $R^2 = 0.94$
- Each point represents a predicted TER value against a measured TER value, with different colors and markers indicating different treatments and timepoints.
- The graph visually compares predicted TER ($\Omega \cdot \text{cm}^2$) against measured TER ($\Omega \cdot \text{cm}^2$), with categories for True Positive, False Positive, False Negative, and True Negative.
Different TML Models Key in on a Similar Set of Cell Features

- Intensity 1 = average minimum intensity per cell
- Texture 1 = 3rd inverse difference moment at 45°
- Texture 2 = 3rd inverse difference moment at 135°
- Shape 1 = Zernike n4-l0 polynomial

What is the biological significance of these metrics?
Different TML Models Key in on a Similar Set of Cell Features

- Intensity 1 = average minimum intensity per cell
- Texture 1 = 3\textsuperscript{rd} inverse difference moment at 45°
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- Shape 1 = Zernike n4- l0 polynomial

What is the biological significance of these metrics?

Local heterogeneity is non-homogenous

\[
Z_4^0
\]

Machine Learning Prediction of VEGF Ratio from WIPP Single Cell Features

Summary of Algorithm Regression Errors

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>VEGF Ratio Root Mean Squared Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNN-F: Deep Learning Neural Network</td>
<td>1.01</td>
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<tr>
<td>RF: Random Forest</td>
<td>1.45</td>
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<tr>
<td>MLP: Multi-Layer Perceptron</td>
<td>1.47</td>
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<tr>
<td>L-SVM: Linear Support Vector Machine</td>
<td>1.59</td>
</tr>
<tr>
<td>PLSR: Principle Least-squares Regression</td>
<td>1.65</td>
</tr>
<tr>
<td>RR: Ridge Regression</td>
<td>1.84</td>
</tr>
</tbody>
</table>

The dashed line represents a perfect prediction between the measured and predicted VEGF ratios.
Results not discussed

• Results confirmed in additional donors…
  • iRPE from 5 albino patients: verify QBAM measurements on iRPE with diagnosable differences in pigmentation
  • iRPE from 2 healthy donors: to verify that QBAM did not impact iRPE maturation
  • Clinical-grade iRPE from 3 AMD donors (and 2 or 3 clones from each of 3 donors): to verify AI predictions from QBAM worked for iRPE from AMD patients
  • Using AI to predicting donor identity from QBAM images
    • FDA requires STR phenotyping of for manufactured autologous cell therapies
Conclusions

- Weekly QBAM imaging did not impact iRPE maturation (non-invasive)
- Unprocessed QBAM could predict iRPE function: TER & VEGF ratio
- DNN of unprocessed QBAM images more accurate than segmentation-TML
- Very important that training set have good & bad samples
  - Time: early timepoints less mature, later timepoints more mature
  - Treatments: HPI4 inhibits RPE maturation, aphidicolin promotes
- BALANCE: If you only feed the AI great samples, then it will predict that the test samples are great
Dissemination

  • https://doi.org/10.1172/JCI131187

• QBAM:
  • SQuIRE: Micromanager plugin to collect images
    • Github: https://github.com/Nicholas-Schaub/SQuIRE
  • CARPE: ImageJ plugin to convert bright-field microscope images into absorbance images
    • Github: https://github.com/Nicholas-Schaub/CARPE

• Data: https://isg.nist.gov/deepzoomweb/data/RPE implanted
  • iRPE from healthy donors
    • Healthy1, live RPE, broadband (232 GB)
    • Healthy2, live RPE, narrowband (291 GB)
  • iRPE from 3 AMD patients (4 GB): 2 or 3 clones per donor, cells were fixed
  • iRPE from 5 albino patients (36 GB): Cells were fixed
  • QBAM and ZO-1 fluorescence images from segmenting routines (4 GB)
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