Time-of-Flight SIMS Imaging Identification of Individual Cells in Heterogeneous Cultures

Christopher A. Barnes, Jeremy Brison, David G. Castner, Buddy D. Ratner
University of Washington Department of Chemical Engineering

Statement of Purpose: Current tissue engineering approaches employ the creation and subsequent implantation of both seeded and unseeded scaffolding biomaterials. Often, autologous cells are harvested by biopsy, cultured *in vitro* to expand cell numbers, seeded on scaffolds and then reimplanted. However, the phenotypic purity and identity of these cells (especially in the case of stem cell implementation strategies), during the culture period, is often difficult to assess. Here, we show that imaging time-of-flight secondary ion mass spectrometry (ToF SIMS) can be used to distinguish mammalian cell types in heterogeneous cultures. Primary rat esophageal epithelial cells (REEC) were cultured with NIH 3T3 mouse fibroblasts on tissue culture polystyrene (TCPS) and freeze-dried. C_{60} etching improved the ToF SIMS image quality and principal component analysis (PCA) and partial least-squares discriminant analysis (PLSDA) were used to identify peaks whose contributions to the total variance in the model were due to either of the two cell types or the substrate. Using PLSDA, unknown regions of cellularity which were otherwise unidentifiable were able to be classified with the control data. Using the results from the loadings in the PLSDA model, peaks were selected which were indicative of the two cell types and ToF SIMS images were created and overlaid which showed the ability to use this method to distinguish features visually.

Methods: Sample substrates were created using a heated 8 mm biopsy punch in a sterile environment to create TCPS disks used for culturing. REECs were seeded at a low density in serum free EpiLife (Cascade Biologics) media and incubated for 24 hours at 37^0C in 5% CO_2. NIH 3T3 fibroblasts were seeded onto the REEC cultures using DMEM + 10% FBS and the samples were cultured for a further 24 hours (37^0C, 5% CO_2). Samples were fixed in 10% neutral buffered formalin solution for 2 hours at 4^0C and subsequently swirled briefly in dH_20 (3x). Finally, samples were submerged in liq. N_2 and lyophilized overnight. In all, samples were created for each of the individual cell types as controls in addition to the mixed cultures. ToF SIMS images were acquired using an ION-TOF TOF.SIMS 5-100. The analysis beam used for this study was a pulsed 25 keV Bi_3^+ and the etching beam was a 10 keV C_{60}^+. The Bi_3^+ beam was set in the burst alignment mode for imaging (long pulses, nominal mass) and was typically rastered over a 500 × 500 μm^2 area. Etching time points were considered (30, 60 and 90 seconds) and 30 seconds etching was chosen for analysis due to increased retention of features in the images. Averaged images were calibrated to CH_3^+, NH_4^+, $C_2H_3^+$, and $C_5H_{12}N^+$ for the positive ion images. Regions selected around areas that represented cells in each of the images (from both controls and the mixed culture surface). Spectra were created from these regions and multivariate analysis was performed using the region spectra from the controls. Region spectra from the mixed cultures were then classified using principal component analysis (PCA) and partial least-squares discriminant analysis (PLSDA) as part of the PLS Toolbox v4.0 (Eigenvector Research, Inc.) for Matlab.

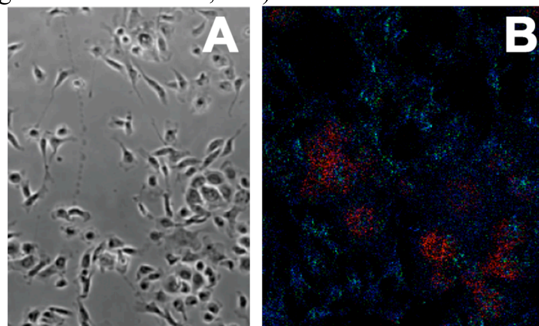

Figure 1: Rat esophageal epithelial cells (REEC) and NIH 3T3 fibroblasts cultured together on TCPS disks. (A) Brightfield image of the mixed cultures. (B) ToF-SIMS image overlay of specific peaks: phosphocholine at m/z 184 ($C_5H_{15}NPO_4^+$) in green, cholesterol at m/z 369 ($C_{27}H_{45}^+$) in red, and potassium in blue to show that cells remained intact and did not burst in the sample preparation.

Results: C_{60} etching times were tested and 30 seconds etching was chosen due to the removal of surface contamination that resulted in the improved retention of image features. To compare the ability to classify regions from the mixed cultures with the calibration data, PCA was performed for both the etched and non-etched samples. It was found that classification was improved when regions from the etched surfaces were input into the PCA model built from the etched calibration data. Using PLSDA, each of the unknown cellular regions was able to be classified with either the REECs or the 3T3s which was an improvement over the PCA classification method. Finally, the loadings from the PLSDA results were used to choose identifiable membrane fragments which could be two-dimensionally mapped to show the locations of the two cell types within the mixed cultures.

Conclusions: In conclusion, this study demonstrates a novel application of ToF SIMS imaging to identify differences in surface regions in mixed cell populations using a relatively simple sample preparation method. Etching with C_{60} for 30 seconds improved the overall ability of the method to discriminate between features on the surfaces. Both the principal component analysis and the classification with partial least-squares discriminant analysis were improved with the etching step. Finally, the results were used to create images which show the visual localization of the two cell types within the mixed cultures.