RRScell method for automated learning immune cell phenotypes with immunofluorescence cancer tissue

Alvason Zhenhua Li¹, *, Karsten Eichholz¹, Anton Sholukh¹, and Lawrence Corey¹,2,3,4

¹Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA
²Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA
³Department of Laboratory Medicine, University of Washington, Seattle, WA 98195, USA
⁴Department of Medicine, University of Washington, Seattle, WA 98195, USA

*Corresponding author (email: alvali@fredhutch.org)

ABSTRACT

Multiplexed immunofluorescence tissue imaging enables precise spatial assessment of protein expression in medical resection specimens. However, tissue sections are stained with a mixture of antibodies, DNA and RNA markers, the detection of weak or broken edges due to fluorescent membrane staining artifacts between touching or overlapping cells is a long term studied problem, and is an active research topic in biomedical image analysis. Sometimes detecting these kinds of edges which are even lacking discrimination or judgment by human visual intelligence. We have built a GPU client-server and have developed a hybrid system combining the stochastic random-reaction-seed (RRS) method and deep neural learning U-net to identify cell-membrane accurately and automatically. Furthermore, we have designed a high performance machine-learning AI-pipeline in quantifying spatial distribution of cell phenotypes from tissue images with various complexities, and extract single-cell or even subcellular resolution profiling-map of protein and RNA expression over a million cells tissue section.

Introduction

Tissue imaging of biopsies and resection specimens plays an important role in translational medicine studies. Particularly, the highly multiplexed immuno-histochemistry (IHC) and RNA/DNA fluorescence in situ hybridization (FISH) raise the possibility of deeper analysis for immune profiling, such as cell types (natural killer cells, cytotoxic T cells, etc.). However, the membrane-image quality defection due to the immuno-staining techniques is common and significant. Noises bring obstacles to edge detection because it reduces the contrast of real membrane boundary and also introduce spurious edges due to noisy contrast, as shown in Figure 1. Detecting weak or broken edges due to fluorescent membrane staining artifacts between touching or overlapping cells is a long term studied problem, and is an active research topic in biomedical image analysis. Sometimes detecting these kinds of edges which are even lacking discrimination or judgment by human visual intelligence. Certain stochastic algorithms (e.g. RRS¹) have been shown some promising results, but subtle edges required handcraft parameters for each image dataset².

Chimeric antigen receptor (CAR) T cells, as one of rapid emerging form of genetically engineered “artificial immune cell”, have outstanding therapeutic potential for treating cancers. To study the anti-HIV CAR T cell trafficking into tissues with viral replication by microscopy, we developed an RNAscope- based RNA fluorescent in situ hybridization assay. In order to validate the RNA probes, we need to quantify a large volume of image dataset from in vitro culture and tissues sections among mouse, monkey, and human. However, the membrane-image quality defection due to the sugar-staining techniques is common and significant. Noises bring obstacles to edge detection because it reduces the contrast of real membrane boundary and also introduce spurious edges due to noisy contrast, as shown in Figure 1. Detecting weak or broken edges due to fluorescent membrane staining artifacts between touching or overlapping cells is a long term studied problem, and is an active research topic in biomedical image analysis. Sometimes detecting these kinds of edges which are even lacking discrimination or judgment by human visual intelligence. Certain stochastic algorithms (e.g. RRS [1]) have been shown some promising results, but subtle edges required handcraft parameters for each image dataset.

As shown in Figure 1, the wide variance of intensity across a large tissue slide is one of the obstacles for precise immune profiling in cellular level. In general, a precise singel-cell level segmentation is anchored on both membrane and nuclei detections. However, commonly, the messy zone (dashed line-loop) is missing the nuclei staining Figure 1 (B2) while the membrane straining is in a messy situation Figure 1 (B1). In case of nuclei segmentation, the nuclei is hard to segmented by human eyes because the DAPI straining is not able to provide clear image that many nuclei are stacking together, In case of membrane segmentation, the Furthermore, even The variability of manual annotation between two individual labelers on the
Figure 1. Challenges in analysis of multiplexed immuno-fluorescent images of cancer tissue with wide variance of intensity and noise. (A) is a typical merged immuno-fluorescent stitched image of a whole cancer tissue slide (red color channel is RNA staining, green color channel is membrane staining, and blue color channel is nuclei staining). (B1) is the raw image from membrane staining channel only. (B2) is the raw image from nuclei staining channel only. Here, the messy zone is missing the nuclei staining.

same membrane image, as shown in Figure 1 (C2) The above examples presented in Figure 1 emphasized the challenges. Here, membranes are well-suited to spatially resolved segmentation techniques offering near-single-cell or even subcellular resolution within tissues. Typically, in some cases, nuclei are not well-suited to automated segmentation: the density in some part of tissues is so high that the images of the nuclei overlap with one another, making discrimination of individual nuclei more difficult. So that, we were focused on images with membrane staining in the early stage of pipeline developement. In practical translational researches, in many circumstances, multiplexed immunofluorescence tissue images are lacking membrane straining. Therefore, the AI-pipeline is also equipped with the artificial membrane generation step to work flexibly and consistently on membrane-less images.

Methods
In general, the manual annotation is used for generating the consensus ground truth. However, the performance of human annotators show a high degrees of variability in complex fluorescent image in tissue as shown in Figure 2(C).

Automated ground truth generation
The major drawback of supervised machine-learning techniques is that the quality of the results depends upon the amounts and quality of manually annotated training data. Training data is generated by manually outlining individual membrane, it is a tedious process. The burden of manual annotation can be reduced by semi-supervised/non-supervised approaches.

Random-reaction-seed strategy
Auto ground truth generation: Beside manual annotation as the ground truth, we have applied the stochastic RRS method\(^1\) to generate high quality ground truth automatically, as shown in Figure 2(E). The accuracy of auto ground truth from RRS is ensured by handcraft parameters for selected image dataset. Auto encoder-decoder net: We have built an auto encoder-decoder network based on the U-Net\(^2,3\) architecture, which is a robust convolutional network in biomedical image segmentation with limited training dataset.

Auto cell-type detection: As shown in Figure 3, we have developed a workflow for phenotype validation cell-by-cell.
Figure 2. Automated ground truth generation in a sample image from cancer tissue. (A) is the merged image of multiplexed. (B) is raw nuclei channel. (C) are the membrane annotations from two experts (red color from one expert, blue color from another expert) on the same membrane image. (D) is the raw nuclei channel. (E) is the dynamic tracing map on the membrane image by the Random-Reaction-Seed method, where the random initial seed is denoted by blue star, the search chain is denoted by red-circle. (F) is the traced membrane profile by RRS, the green-color is the traced membrane plotted on the raw membrane image (hot-color-map).
Figure 3. Scheme of automated cell phenotype detection. Here, an example of 5-plex image is presented: the initial step is the validation of real single cell based on both membrane and nuclei features, and a phenotype detection step will be followed in condition (RNA1, RNA1, and IHC1).
Results

Evaluation of accuracy and efficiency
There is no objective ground truth available for multiplexed immunofluorescence images. To assess the accuracy of our method we compare the results with the manual measurement.

As demonstrated in Figure 4(E), the accuracy of RRScell method is equivalent to manual annotation. Furthermore, due to DAPI staining defect in high intensity zone beyond human visual approach, so that both experts are avoiding these messy areas, as shown in Figure 4(C, D), one significant advantages of RRScell is able to tracing more cells based on its powerful combination of both membrane and nuclei as detection, as shown in Figure 4(B). The correct detection of cellular membrane is verified by the nuclei plot in Figure 4(A). As shown in Figure 4(E), the polar plot of bar-chart, where the polar axis is the similarity score, the blue-color bars are corresponding to the cells (blue dots) in the blue box-plot while the green-color bars are corresponding to the cells (green dots) in the green box-plot. In order to demonstrate the variability of manual annotations, the blue-color bars are sorted in descending order, and then the green-color bar is placed by tracking the similar cellular physical location. So that the similarity of each cell annotated by two experts are displayed in a contrast way. The similarity score of each cell membrane profile is calculated by the IoU (intersection over union).

Extension to tissue images without membrane straining
In practical translational researches, in many circumstances, multiplexed immunofluorescence tissue images are lacking membrane straining. Therefore, the AI-pipeline is also equipped with the artificial membrane generation step to work flexibly and consistenly on membrane-less images.

Discussion
We present a robust, open-source, pipeline for immune cell profiling in spurious-edge-tissues of translational medical research. This machine-learning solution builds upon RRS automated ground truth generation. The house-made automated machine learning pipeline is automatic scaling up from 4 to 5, or highly multiplexed immuno-fluorescent images. As shown in Figure 6, a five-multiplexed tissue is evaluated by different gating conditions.

The results presented on above figures demonstrated that satisfactory segmentation can be obtained using RRScell. The performance analyses demonstrate that RRScell generates segmentation equal or surpass the accuracy of segmentations obtained using manual membrane profile. Significantly, accurate segmentations were obtained for a range different kinds of images.

In conclusion, RRScell, as a hybrid system combining HMM-based RRS and deep neural learning network, has demonstrated the effectiveness and robustness for precise quantification of immune cell phenotypes in large stitched images taken from noisy multiplexed immunofluorescence cancer tissue, which is becoming an increasingly useful tool for biomedical researches owing to its ability to profiling cellular phenotyping in cancer resection tissues with various complexities. Furthermore, the imaging cytometry and AB-zone-analysis programs inside RRScell has emerged as very promising tools in ongoing high-dimensional immuno-straning of cancer tissue.

References
1. Li, A. Z., Corey, L. & Zhu, J. Random-reaction-seed method for automated identification of neurite elongation and branching. Sci. Reports 9, 2908, DOI: 10.1038/s41598-019-39962-0 (2019).
2. Ronneberger, O., Fischer, P. & Brox, T. U-net: Convolutional networks for biomedical image segmentation. https://arxiv.org/abs/1505.04597 (2015).
3. Falk, T. et al. U-net: deep learning for cell counting, detection, and morphometry. Nat. Methods 16, 67–70, DOI: 10.1038/s41592-018-0261-2 (2019).

Acknowledgments
This work was partially supported by the National Institutes of Health [grant number UMI A126623]. The authors would like to thank Drs. Sijie Sun, and Mindy Miner for their constructive comments to improve the quality of the paper. All the raw fluorescent images are provided by Karsten Eichholz in the HSV Vaccine Lab at the Vaccine and Infectious Disease Division of Fred Hutchinson Cancer Research Center.

Author contributions statement
A.Z.L. conceived the algorithm, analyzed the results and wrote the manuscript; E.K. coordinated and developed the chimeric antigen receptor T cells investigations; A.S. coordinated the setup of GPU client-server; L.C. supervised the cancer tissue studies.
Figure 4. Performance of RRScell method. (A) Membrane profile from RRScell is plotted on the nuclei image. (B) Membrane profile from RRScell is plotted on the membrane image. (C) Manual membrane profile from Expert-1 is plotted on the membrane image. (D) Manual membrane profile from Expert-2 is plotted on the membrane image. (E) The box-plot on the top is the evaluation of accuracy among three different methods, the red-square-point inside each box is the mean value. Comparison between membrane-detection methods: the blue-color box is the similarity between RRScell and expert-1, the blue-color box is the similarity between RRScell and expert-2, the black-color box is the similarity between two experts. The polar plot of bar-chart on the upper-right is the evaluation of similarity of each cell between experts: each blue-color bar is corresponding to each dot in the blue-color box-plot, here, the bars are sorted in descending order. The box-plot on the lower-right is the evaluation of efficiency, the efficiency of RRScell is significantly better than the manual process (>30 folds difference in processing time).
Figure 5. Cell phenotype analysis of 4-plex immuno-fluorescent image. The left panel is the raw stitched cancer tissue. The right panel is the composition of 4 phenotypes from AI inferencing (the pie-chart on the corner displays the cell-type ratio over 200,000 cells in the tissue).
**Figure 6.** Cell phenotype analysis of 5-plex immuno-fluorescent image. The left panel is the raw stitched cancer tissue. The right panel is the composition of 5 phenotypes from AI inferencing (the pie-chart on the corner displays the cell-type ratio over 200K cells in the tissue).

**Data availability statement**

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

**Additional information**

**Competing interests:** The authors declare no competing interests.