# 1 Abstract

# 2 INTRODUCTION

Sepsis is a serious and often deadly condition that occurs when the body’s response to infection causes inflammation throughout the body. Despite efforts to improve recognition and treatment, sepsis and its most severe form, septic shock, still have high mortality rates. Currently, the diagnosis and prognosis of sepsis and septic shock primarily rely on clinical criteria, and while multiple biomarkers have been studied, none of them are accurate enough to be used alone in clinical practice. To identify patients who would benefit the most from targeted interventions, new tools are needed to accurately characterize the various pathogen and host factors that influence outcomes for patients with sepsis and septic shock. One aspect of sepsis to consider is the host immune response, which is complex and often involves a proinflammatory phase with the release of cytokines such as TNF-α and IL-6. T cells, particularly CD8+ T cells or cytotoxic T lymphocytes (Tc cells), play a vital role in sepsis[**He2021Immune?**](#ref-He2021Immune). T cells produce INF-γ and IL-17 within the first 24 hours of sepsis, which enhance innate immune function[**Kasten2010CELLS?**](#ref-Kasten2010CELLS), and naive pathogen-specific CD8 T cells are important for infection clearance and the generation of memory CD8 T cells[**Danahy2016Clinicala?**](#ref-Danahy2016Clinicala). However, T cell activation can also lead to exhaustion in sepsis, resulting in decreased immune function [@].

Optical microscopy is a commonly used technique for studying micro-objects, but it has limitations and can alter cellular processes through the use of fluorescent stains and molecular markers. Holotomography is a method that avoids these issues by offering high sensitivity and the ability to present images in absolute values without the need for invasive interventions or sample preparation[**Vasilenko2017Opportunities?**](#ref-Vasilenko2017Opportunities). It also allows for the real-time study of cellular processes and can provide measurements of nuclear refractivity, which is the degree of chromatin decondensation and can be used as a quantitative criterion for evaluating the dynamics of nuclear proteins and chromatin in normal and pathological conditions[**Vasilenko2017Opportunities?**](#ref-Vasilenko2017Opportunities).

In this study, we hypothesized that the cell refractive index of sepsis patients would differ from that of a healthy group and that this would allow us to distinguish between sepsis and healthy individuals based on their T cell refractive index. We used holotomography to evaluate T cell refractive index in sepsis patients and healthy controls and to determine whether T cell refractive index could be used to distinguish between the two groups. Our results suggest that T cell refractive index may be a useful tool for distinguishing between sepsis and healthy individuals and for monitoring the immune response in sepsis.

# 3 RESULTS

## 3.1 Longitudinal [[3D RI Tomogram]] [[CD8+ T cell]] in sepsis patients

We acquired [[3D RI Tomogram]] on [[CD8+ T cell]]s from people with sepsis and controls to define the range of cell states present in these subjects, to identify differences in cell-state composition between groups, and to detect immune signatures that distinguish sepsis from the normal immune response to bacterial infection (Fig. 1) supplementary.

Optical section of the cell in each group were observed. The dry mass, and mean RI values of optical section of the cell were higher in sepic shock group than in any other groups.

## 3.2 Different [[3D RI Tomogram]] distribution between septic shock status and healthy control

We examined whether the distribution of refractive index (RI) differs between septic shock patients and healthy patients. To analyze the spatial distribution of RI, we divided two-dimensional cell images into eight shells based on optical sections and numbered them from 1 to 8, starting from the center and moving towards the periphery. We also classified RI into high (RI > 1.38), medium (1.36 < RI < 1.38), and low (1.345 < RI < 1.36) categories and evaluated the density of each category in each shell (Fig. ??A). In septic shock, the distribution of high RI was higher in all shells (p-value ##) and the elbow point of density was slightly more peripheral compared to other groups (Fig. ??B). As a result, the density of medium RI was relatively lower (Fig. ??B). Additionally, overall, the elbow was more peripheral in septic shock (Fig. ??D). Generally, high RI is associated with an increased proportion of the nucleus, which may indicate increased nuclear size and transcriptional activity as well as larger cell size in septic shock.

## 3.3 Deep Learning Models for Sepsis Diagnosis and Prognosis Using [[3D Refractive Index of Cell]]

We observed that the distribution of RI varies according to the severity of sepsis, and we sought to determine whether the distribution of RI itself can be used to diagnose and predict the prognosis of sepsis. To this end, we developed deep learning models that can distinguish between sepsis and healthy individuals (diagnosis model) and between survivors and non-survivors in septic shock (prognosis model). When only a single cell was considered, the average AUROC for both the diagnosis and prognosis models was ## and ##, respectively. However, when we generated an ensemble model using multiple cells, we found that the AUROC was almost always greater than 99% when three or more cells were used (Figure ??A, B). These results show that our approach performs significantly better than established clinical indicators such as NEWS, MEWS, SOFA, and qSOFA, with AUROC values of ##, ##, ##, ## for diagnosis and ##, ##, ##, ## for prognosis, respectively. Furthermore, when we examined the AUROC with random sampling to account for possible selection bias, we found that the range of AUROC decreased as the number of cells increased (Figure ??C, D).

## 3.4 [[3D RI Tomogram]] correlates with clinical value in sepsis patients

RI분포만을 가지고 실제 환자의 진단이나 예후 예측이 가능하여 실제 clinical feature들과의 관계도 확인해보았다. 이러한 RI distribution과 임상 지표인 SOFA score 및 laboratory test인 WBC, neutrophil, lymphocyte, CRP, 또한, cytokine level과의 association을 확인하였다.

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## 3.5 Visual explanation of the deep learning model

We applied the [[Grad-CAM algorithm]][**Selvaraju2020GradCAM?**](#ref-Selvaraju2020GradCAM) to understand which regions of the [[cell images]] were most important for the deep learning model’s prediction. Figure ?? shows representative examples of cell images with accompanying saliency heatmaps, which highlight the features that had the greatest influence on the model’s prediction. By examining the heatmaps, we can see that the model was basing its prediction on features within the nucleus and cytoplasm of the cells. The heatmaps also varied between different time points, indicating that the model could recognize changes in the relative importance (RI) of these features over time.

# 4 DISCUSSION

RI ~ function of the cell RI ~ nucleus epigenetic change

T1에서 보이는 변화는 CD8+ T cell의 activation으로 인한 변화이지 않을까? T cell의 활성화가 central DNA pattern과 increase nuclear size로 대변된다[**Gupta2012Developmental?**](#ref-Gupta2012Developmental).

기본적으로 cytokine release는 cell에서 일어나는데 이러한 cell의 변화가 먼저 생기고 cytokine release가 일어나는 것이다. 따라서 cell morphology를 측정하는 것이 더 빠르게 면역 반응을 볼 수 있는 방법이다.[@] Importantly, circulating substances might not represent the cellular response very well and other types of markers might be more relevant[**Cohen2015Sepsis?**](#ref-Cohen2015Sepsis).

Takes only 1 ml of blood and a 3-hour turnaround time. 다른 것들은 시간이 많이 걸린다.

In a single droplet of blood, there are many T cells present, and using only three or more images, we obtain almost 100% performance.

The sample size was small, however, we recruited patients longitudinally.

In conclusion, our approach represents a flexible strategy that can be easily used to detect the presence of T cells even help to diagnosis and predict prognosis of sepsis.

# 5 REFERENCE

# 6 MATERIALS AND METHODS

## 6.1 Study samples and clinical adjudication

The septic shock cohorts comprised subjects with septic shock who presented to the ED at the Severance hospital in Korea. Informed consent was obtained from subjects or their surrogates. To define the trajectory of septic shock, for each individual, data were collected and blood samples were drawn with EDTA Vacutainer tubes (BD Biosciences) near septic shock diagnosed ([[T1]]), septic shock was resolved ([[T2]]), and before discharge ([[T3]]). The study was approved by the institutional review board of Severance Hospital, Yonsei University Health System, Seoul, Korea, and all patients provided informed consent (IRB No.4-2021-1236).

The study cohort was enrolled in the ED at the Severance Hospital from April 2022 to June 2022. They consisted of people with septic shock, defined by Sepsis-3 consensus definitions[@], specifically, (1) with suspected infection, (2) 2 or more criteria of qSOFA, which consists of respiratory rate >= 22/min, altered mentation, and systolic blood pressure <= 100 mmHg, were satisfied, (3) an acute change in total SOFA score[@] ≧ 2 points consequent to the infection. Study samples were collected within 12 h of subject’s arrival at the ED. Patients with followings were excluded: (1) age under 19, (2) pregnant or lactating, (3) active cancer status (4) acute stroke, (5) acute cardiovascular disease, (6) acute burn, (7) acute gastrointestinal bleeding or within the last three months of bleeding, (8) taking immunosuppressive drugs after organ transplantation, (9) taking immunosuppressive drugs for autoimmune disease, (10) immunodeficiency conditions that have been diagnosed before, or that have CD4 cell counts below 350 G/L, (11) neutropenia (neutropenia < 500 G/L) or if the neutrophils were 500-1000 G/L due to chemotherapy and were expected to decrease, (12) diagnosed with adrenal dysfunction, (13) prescribed steroid equivalent to or greater than 0.5 mg/kg/day prednisone, (14) active tuberculosis, (15) cystic fibrosis, (16) post-traumatic, (17) who needed immediate surgery, and (18) the state of Do-Not-Resuscitate (DNR). The study was approved by the institutional review board of Severance Hospital, Yonsei University Health System, Seoul, Korea, and all patients provided informed consent.

The healthy cohorts consisted of a total of 20 volunteer healthy patients as a control group to compare with a cohort of septic shock patients.

## 6.2 Isolation PBMCs from whole blood

Peripheral whole blood was collected in EDTA tubes and processed fresh via Ficoll-Paque Plus separation (GE Healthcare, 17144002). The blood was first diluted with 5 ml 2 mM EDTA-PBS (Invitrogen, 1555785-038), before 10-20 ml of diluted blood was carefully layered onto 15 ml of Ficoll in a 50 ml falcon tube. The sample was centrifuged at 900g for 30 min at room temperature. The plasma layer was carefully separated and the PBMC layer was collected using a sterile Pasteur pipette. The PBMC layer was washed with three volumes of EDTA-PBS by centrifugation at 500g for 5 min. The pellet was suspended in EDTA-PBS and centrifuged again at 400g for 5 min. The PBMC pellet was collected, and the cell number and viability were assessed using Trypan blue (The Countess II Automated Cell Counter, ThermoFisher, USA).

## 6.3 Sorting cells

The magnetic activated cell sorting (MACS), a typical method for isolating the cells from a mixed population, was used for cell sorting (MACS from Miltenyi Biotec, Bergisch Gladbach, Germany)[@]. The [[CD8+ T cell]]s were negatively selected by MACS. Isolated cells were kept at 4°C to keep the viability of the cells. 80 μL isolation buffer and 20 μL MicroBeads were added and mixed the cells in the incubator for 15 minutes at a temperature 4°C. By setting the magnetic stand and column for sorting we first equilibrate the column by washing 3mL of the isolation buffer. Next to collect each of lymphocytes, we withdrew the column from the magnetic rack and set up a conical tube. By adding 5mL of isolation buffer, we pumped the solution through the column to extract the final sorted cells from the collection tube.

## 6.4 3D RI tomography setup and data acquisition

We took three-dimensional (3D) refractive index (RI) images of the CD8 T cells with 3-D quantitative phase imaging (QPI) system [@], which is commercialized and dubbed holotomography (HT-2H, Tomocube Inc., Daejeon, Republic of Korea) [@]. The digital micromirror device scans the various illumination angles and the 3D RI tomogram is reconstructed from the sinogram of 2D QPI measurements under the principle of optical diffraction tomography [@].

## 6.5 RI distribution 보는 방법 (shell)

## 6.6 Image preprocessing and data augmentation. (종현 - 아래 주석 참고해서 다음 내용 수정해주세요)

## 6.7 CNN training (종현 - 아래 주석 참고해서 다음 내용 수정해주세요)

## 6.8 Visual explanation

We used Grad-CAM[@] to derive visual explanation by localizing the image area that most influences the decision made by the deep learnig model.

## 6.9 Statistical analysis

We used PR curve and ROC curve to describe the classification ability of [[the deep learning model]]. The PRC was demonstrated to be more informative than receiver-operating curve on imbalanced datasets[@]. We created the PR curve by plotting recall rate (also known as sensitivity) against precision (also known as positive predictive rate) by varying the predicted probability threshold. The ROC curve was created by plotting recall rate against negative predictive rate (also known as specificity). The F1 metric is defined as harmonic mean between precision and recall rate, which is calculated as F1 = 2 \_ precision \_ recall/(precision + recall). The 95% confidence intervals for sensitivity, specificity, positive predictive rate, and negative predicted rate were calculated by the Clopper-Pearson method[@]. We plotted PR curve and calculated the AUPRC with R package PRROC[@] (version 1.3.1). We plotted the ROC curve and AUROC with R package pROC (version 1.10.0)[@]. Statistical analysis was conducted with R software (version 3.4.3)[@]. We used AUPRC as the primary outcome to measure the performance of [[the deep learning model]]. We used precision, recall rate and F1 score when comparing the classification ability of CRCNet with endoscopists.

## 6.10 Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

# 7 Data availability

# 8 Code availability

# 9 Reference

# 10 Acknowledgements

# 11 Competing interests

# 12 Additional information