A morphology and secretome map of pyroptosis

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Abstract

Pyroptosis represents one type of Programmed Cell Death (PCD). It is a form of inflammatory cell death that is canonically defined by caspase-1 cleavage and Gasdermin-mediated membrane pore formation. Caspase-1 initiates the inflammatory response (through IL-1β processing), and the N-terminal cleaved fragment of Gasdermin D polymerizes at the cell periphery forming pores to secrete pro-inflammatory markers. Cell morphology also changes in pyroptosis, with nuclear condensation and membrane rupture. However, recent research challenges canon, revealing a more complex secretome and morphological response in pyroptosis, including overlapping molecular characterization with other forms of cell death, such as apoptosis. Here, we take a multimodal, systems biology approach to characterize pyroptosis. We treated human Peripheral Blood Mononuclear Cells (PBMCs) with 36 different combinations of stimuli to induce pyroptosis or apoptosis. We applied both secretome profiling (nELISA) and high-content fluorescence microscopy (Cell Painting). To differentiate apoptotic, pyroptotic and healthy cells, we used canonical secretome markers and modified our Cell Painting assay to mark the N-terminus of Gasdermin-D. We trained hundreds of machine learning (ML) models to reveal intricate morphology signatures of pyroptosis that implicate changes across many different organelles and predict levels of many pro-inflammatory markers. Overall, our analysis provides a detailed map of pyroptosis which includes overlapping and distinct connections with apoptosis revealed through a mechanistic link between cell morphology and cell secretome.

Introduction

Cell death pathways such as apoptosis and pyroptosis are defined canonically via cleaved caspases and specific morphologies.[1–4] Pyroptosis (originally discovered in macrophages) is caspase-1 dependent and forms membrane pores, resulting in nuclear compaction and DNA fragmentation.[5] These membrane pores form through Gasdermin D cleavage and translocation of its N-terminal domain to the cell and mitochondrial plasma membranes, where subsequent pores enable the secretion of pro-inflammatory markers.[6] Conversely, apoptosis, one form of canonically non-inflammatory cell death, is caspase-3 dependent and causes membrane blebbing, nuclear compaction, and DNA fragmentation.[7] Though defined separately from

pyroptosis, recent research suggests they may have important overlaps. For example, both caspase-1 and caspase-3, in certain cases, are involved in both apoptosis and pyroptosis.[8,9]

With a growing appreciation for the complex intersection of cell death processes[10,11], we sought to acquire and analyze comprehensive representations of the secretome and cell morphology to disambiguate apoptosis from pyroptosis. Furthermore, we focused on pyroptosis because several links between pyroptosis and disease have emerged in recent years. Aberrant regulation of pyroptosis has been implicated in inflammatory disease, cancer, chemotherapy resistance, and autoimmunity.[12,13] Therefore, manipulating pyroptosis with therapeutic agents represents a new, underexplored frontier for many diseases. Unfortunately, incomplete knowledge of pyroptosis limits therapeutic development. To develop new therapeutic agents targetting pyroptosis, the full landscape of the cellular pyroptotic states need to be elucidated and differentiated from other forms of cell death. We begin charting this map of pyroptosis using systems biology.

Systems biology provides tools to measure comprehensive representations of biological processes by measuring holistic features in the same cell.[14,15] There are many different ways to measure systems biology, each providing complementary views into cell function. Scientists have used systems biology to measure cell death complexities. For example, Sato et al. measured gene expression of cells undergoing necrosis (another form of cell death), and apoptosis to understand how genes differentially regulate these pathways.[16] Further, Yu et al. discovered a gene expression signature of pyroptosis in low-grade glioma patients.[17] In general, emerging assays and new approaches for studying complex diseases have enabled more extensive systems biology characterization of cell death.[18–21] While recent work has characterized disease-specific transcriptome differences[22–24], pyroptosis has not been characterized via other systems biology views including high-content imaging and secretome.

High-content microscopy is an emerging systems biology measurement. Microscopy yields robust information that is not captured by single molecule approaches such as sequencing, or the human eye.[25,26] One of the most common high-content microscopy assays is Cell Painting. Cell Painting fluorescently stains the cell's nucleus, mitochondria, endoplasmic reticulum (ER), cytoplasmic RNA, nucleoli, actin, Golgi apparatus, and plasma membrane.[27] From these images, scientists extract high-content representations using a bioinformatics approach known as image-based profiling.[28] These high-content representations have mostly been used for drug discovery[29,30], but recent research has applied this approach for basic research studies, such as predicting cell health, toxicity, and cancer cell resistance.[30–34] While the most common image-based profiling approach is to aggregate single-cell measurements per well, single-cell approaches enable more systematic analyses and are becoming increasingly common.[35] For example, Schorpp et al. developed a machine-learning approach to predict apoptosis and necrosis from Cell Painting data at single-cell resolution.[36] Similar advances in ELISA technologies have enabled the simultaneous acquisition of hundreds of secreted markers to form systems biology representations of the secretome, and recent technology allows the simultaneous measurement of secretome and cell morphology from the same wells.[37,38]

In this study, we applied Cell Painting and nELISA (Nomic Bio - 187 secretome markers) to profile cell morphology and secretome representations of pyroptosis and apoptosis. We modified the Cell Painting assay to swap the cytoplasmic RNA stain with a marker for cleaved N-terminal Gasdermin D protein, which is the canonical marker of pyroptosis pore formation.[6,39] We applied this assay to Peripheral Blood Mononuclear Cells (PBMCs) perturbed with 36 chemical agents at different doses that induced or inhibited either apoptosis or pyroptosis (e.g., lipopolysaccharide, nigericin, flagellin, hydrogen peroxide, etc.). We confirmed pyroptosis and

apoptosis activity through the presence of canonical secreted markers, but individual pyroptosis treatments induced a complex secretome landscape. We also conducted extensive comparative analysis through training hundreds of machine-learning models to uncover distinctive cell morphology patterns associated with pyroptosis. Using cell morphology data, we successfully predicted levels of biologically significant pro-inflammatory cytokines such as IL-1β and TNF-α. We discovered key differences in nuclei morphology and Gasdermin D distribution, but pyroptotic cells showed different morphologies in all profiled organelles. Deep learning networks of single-cell morphology profiles demonstrated accurate differentiation between pyroptotic and apoptotic cells. In summary, our analysis revealed both shared and unique responses in pyroptosis and apoptosis and established a mechanistic link between cell morphology and the cell secretome.

Results

Experimental design and cell death process validation

We induced either apoptosis or pyroptosis by treating PBMCs with 36 different combinations of pyroptosis and apoptosis-inducing and inhibiting agents (**Supplemental Table 1**). To isolate pyroptosis, we treated cells with LPS, flagellin, or a mixture of LPS and nigericin.[40–45] To isolate apoptosis, we treated cells with hydrogen peroxide (H2O2) and thapsigargin.[44,46–49] In addition, we also incubated some wells with apoptotic and pyroptotic inhibitors prior to treatment. We used disulfiram to inhibit Gasdermin D-mediated pore formation[50–53] and Z-VAD-FMK to inhibit the proteolytic activity of caspases.[42,45,47,50] Combining inhibitors with inducers maximizes our ability to isolate specific cell death pathways. We incubated cells for one hour with an inhibitor/control, followed by six hours of cell death induction stimuli at varying dosages. We included six to eight replicate wells of each inhibition/induction combination (36 combinations) within one 384-well plate. For a complete plate map, see **Supplemental Figure 1**. We applied a modified Cell Painting assay paired with secretome profiling to measure two systems biology representations of PBMCs treated with this specific combination of apoptotic and pyroptotic agents (**Figure 1**).

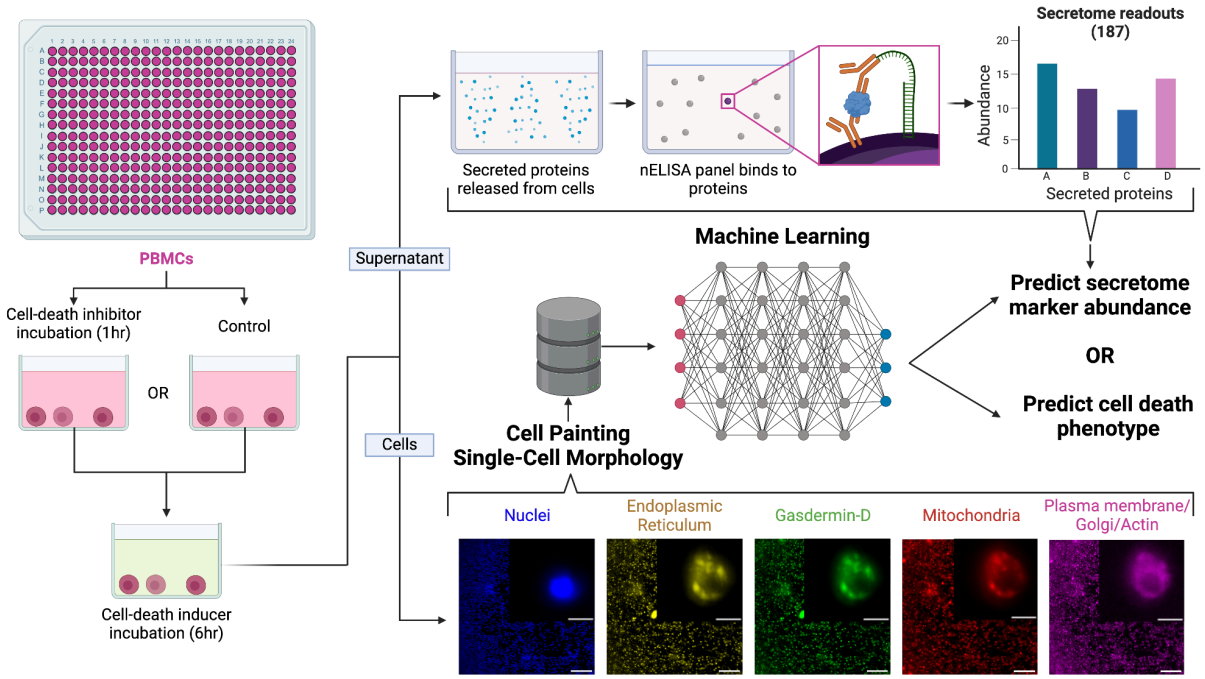

Figure 1. *nELISA and imaging workflows.* Experimental workflow for Peripheral Blood Mononuclear Cells (PBMCs) treated with cell death inhibitors and inducers. We use the supernatant for the nELISA panel to identify the abundance of 187 secreted proteins and the fixed cells for a modified Cell Painting assay, which stains for six organelles and cleaved Gasdermin D. We applied different machine learning pipelines to predict individual secretome marker abundance and single-cell cell death phenotype. The scale bars in the representative Cell Painting image represent 5μm (inset) and 100μm (full image).

We confirmed that pyroptotic treatments were eliciting an inflammatory pyroptotic response via the secretion of canonical markers IL-1β and TNF-α (**Fig. 2A; Supplemental Figure 2A**).[39,54–58] We also confirmed that apoptotic treatments elicited the canonical secretome response by measuring the CCL24 and IL-1β secretion **(Supplemental Figure 2B)**. We applied Uniform Manifold Approximation Projection (UMAP)[59] which showed replicate wells grouping together and apoptosis clustering separately from pyroptosis, which formed two clusters: One cluster driven by LPS and flagellin and the other by LPS+nigericin **(Fig. 2B; Supplemental Figure 2C**). Furthermore, we observed expected enrichment of other canonical cell death markers. For example, pyroptosis inducers selectively increased levels of IL-18 and IL-6, while apoptosis inducers selectively increased CCL24, CCL13, and IL-2 (**Fig. 2C**).[60–72] We applied an analysis of variance (ANOVA) to the 187 secretrome markers and observed 37 differential secretome markers between apoptosis and pyroptosis (Tukey's Honestly Significant Difference [HSD] test $p < 0.05$). Hierarchical clustering of these markers showed clear and consistent differences for each treatment group **(Fig. 2D; Supplemental Figure 3)**. Many of these differential secretome markers have not yet been reported in the literature, including CCL24, Osteopontin, and IL-2. See **Supplementary Table 2** for a full list of significantly different secretome markers across healthy, apoptotic, and pyroptotic cells.

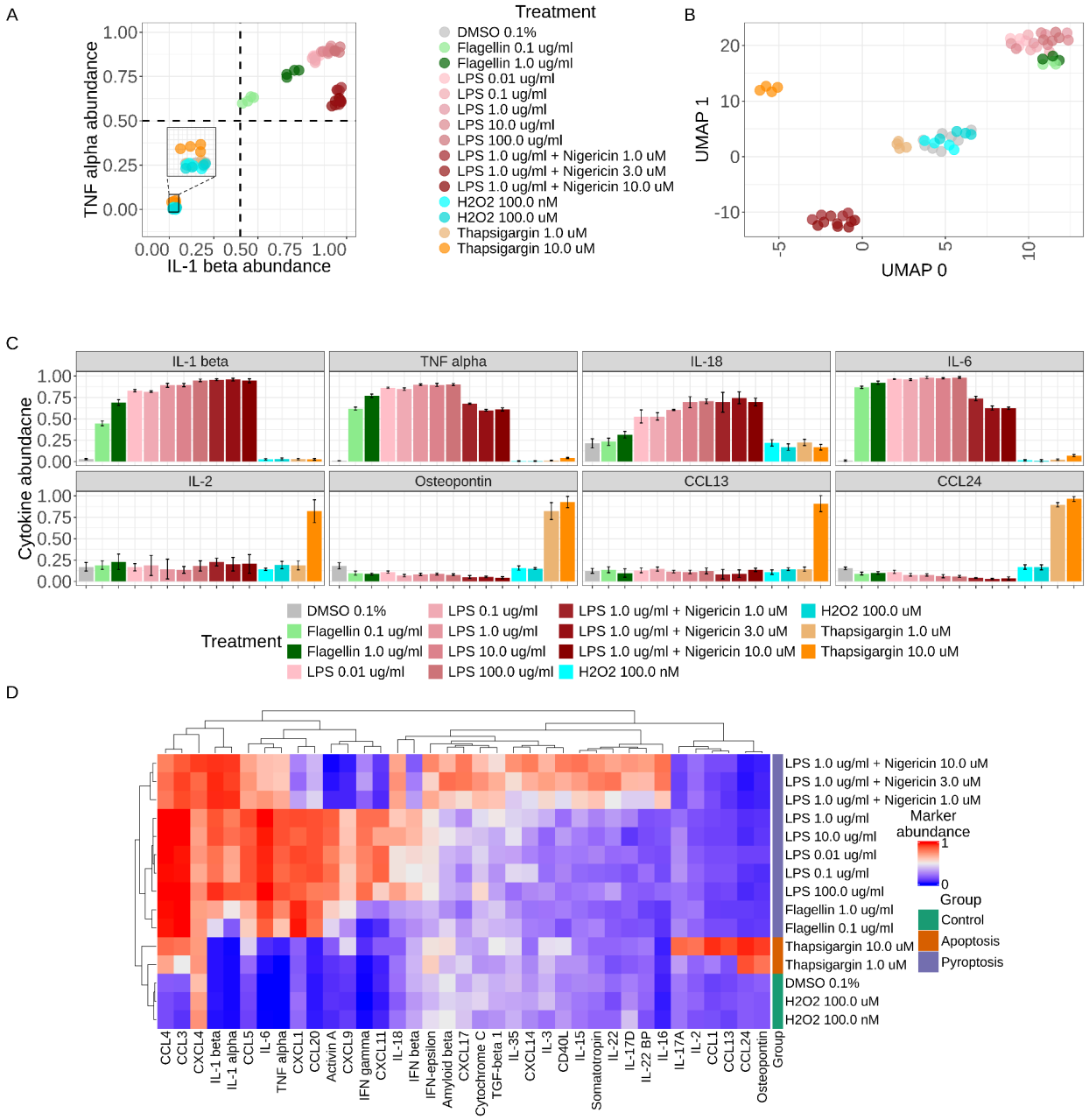

Figure 2. *Pyroptosis and apoptosis have distinct secretome profiles.* **(A)** Abundance of IL-1β and TNF-α secretions, which are canonical pyroptosis markers. Axis values are min-max scaled abundance measurements from the nELISA assay. The dotted lines represent gates used as ground truth for treatments that induce or do not induce pyroptosis. **(B)** Applying Uniform Manifold Approximation Projection (UMAP) to secretome data reveals differential clustering by specific cell death-inducing agents and controls. **(C)** Min-max normalized abundance of secreted proteins known to be associated with pyroptosis and apoptosis. Whiskers on bars represent standard deviation across well replicates. **(D)** Select secreted secretome marker values (analysis of variance [ANOVA] per feature; selected features with $p < 0.05$) across treatments show differential distinct secretome markers abundance for apoptotic, pyroptotic, and control treatment cells.

Cell Painting reveals distinct single-cell morphologies across pyroptotic and apoptotic treatments

We also performed high-content microscopy in the same wells where we measured the secretome profiles. Specifically, we applied a modified Cell Painting assay to include a stain for N-terminus cleaved Gasdermin D (**Fig. 3A**). We used CellProfiler[73] as part of an image analysis pipeline for quality control (QC), illumination correction, cell segmentation, and single-cell morphological feature extraction. We then used CytoTable[74] and pycytominer[75] to perform image-based profiling for single-cells (see methods for details) (**Supplemental Figure 4**). In total, we measured 2,907 morphology features in 8,318,724 single-cells (439,316 apoptosis, 3,578,372 pyroptosis, and 4,301,036 controls). Downsampling these cells and applying UMAP to feature-selected single-cell morphology features did not reveal a clear separation between pyroptotic, apoptotic, or control treatments (**Fig. 3B; Supplemental Figure 5**). Nevertheless, we applied an ANOVA and identified many differential morphology features across pyroptotic, apoptotic, and control treatments **(Supplemental Table 3).** We observed 402 features that were consistently different in all comparisons, but many differential features were unique to that specific comparison (**Fig. 3C**). For example, we observed 67 unique differential morphology features between pyroptosis and control group cells, with most morphology changes relating to nuclei and AGP (**Fig 3D**). A previous cyro-EM study identified co-localization of Golgi apparatus vesicles with cytoskeletal filaments during pyroptosis.[76] Contrastingly, we identified 367 differential morphology features between apoptosis and control group cells, with most morphology changes from the mitochondria and nuclei (**Fig. 3E)**. When comparing the apoptosis with pyroptosis group cells, we identified 98 differential features across all organelles, which indicates broad differences (**Fig 3F**). Of the 1,199 feature-selected morphology features, 402 were differential (34%) between all of the pyroptotic, apoptotic, and control group comparisons (**Fig 3G**). We observed a total of 1,027 differential features in any comparison (86%) indicating widespread morphological changes that occur during cell death.

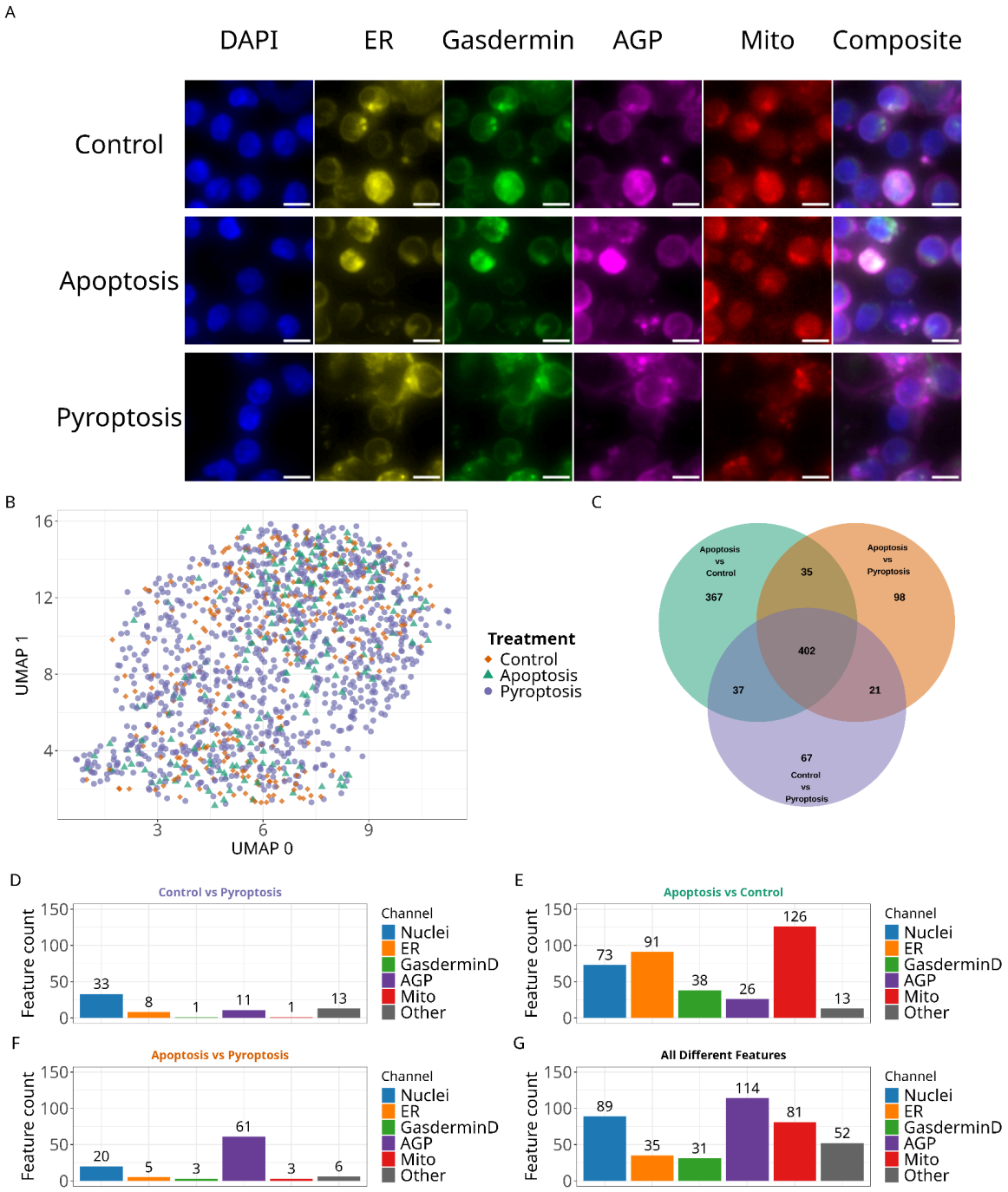

Figure 3. *Cell Painting distinguishes single-cell morphology differences across different cell death types.* **(A)** PBMC montage of Cell Painting. The scale bar represents 5µm. Each row represents single-cells randomly selected to represent each cell death class. Control (top row), apoptosis (middle row), and pyroptosis (bottom row). The composite images are the merged blue, green, and magenta channels, representing the DAPI, Gasdermin D, and AGP channels, respectively. **(B)** UMAP visualization of downsampled single-cell morphologies across selected treatments. **(C)** Venn Diagram of statistically significant morphology features identified by ANOVA and Tukey's HSD post hoc test. Tukey's HSD identified morphology differences across channels for **(D)** pyroptosis vs. control, **(E)** apoptosis vs. control, and **(F)** apoptosis vs. pyroptosis treatments. **(G)** All 420 morphology features that were different in any comparison.

Secretome and morphology profiling provide complementary information for mapping cell death states

We next sought to determine the complementarity of secretome and high-content cell morphology. Because the data are paired (both measurements from the same well), we can directly compare each measurement. We observed high mean average precision (mAP) scores for both morphology and secretome measurements of the same cell death category or treatment although morphology mAP was generally higher (**Fig 4A; Supplemental Figure 6**).[77] mAP measures both consistency of replicate treatments and effect size compared to controls (see methods for details), which indicates that our treatments were generally reproducible and induced large changes.

We next trained 187 logistic regression models with an elastic net penalty to predict individual secretome marker abundances using well-level cell morphology. We found that 20% of the models (40/187) had R^2 performance in the test set greater than 0.8, indicating high performance (**Fig. 4B**). For example, IL-1β is one of the highest-performing models (test set R^2 = 0.98) (**Fig. 4C**). Model performance was particularly low in cases where morphology contributed to a low amount of variance in secretome marker data (**Supplemental Figure 7A**). Globally, we predicted the secretome only marginally better than the shuffled baseline, which highlights the secretome complexity and the ability to predict only certain secreted markers from cell morphology (**Supplemental Figure 7B**). Despite low predictive power globally, specific markers such as IL-6, showed high model performance (test set R^2 = 0.98) (**Supplemental Figure 7C**). However, many other secretome markers were not predicted well, such as IL-11 (test set R^2 = -0.27) (**Supplementary Figure 7D**). Importantly, we also do not see high performance in randomly shuffled data, which represents a negative control baseline and confirms that high-performing models can predict secretion of certain secretome markers directly from cell morphology.

We can directly interpret the importance of individual morphology features for making secretome marker predictions. For example, accurate IL-1β predictions rely on many different morphology feature categories, particularly on granularity patterns of mitochondria near nuclei and the correlation of ER distribution with other organelles in the cytoplasm **(Fig. 4D)**. Other high performing secretome markers were influenced by diverse feature sets as well **(Fig. 4E; Supplemental Figure 8)** The majority of secretome markers could not be predicted by morphology, but most morphology features contributed to some secretome marker predictions (**Supplemental Figure 9**). The link between morphology features and secretome markers present mechanistic hypotheses about morphological changes that occur either as a cause or consequence of the processes that lead to specific marker secretion.

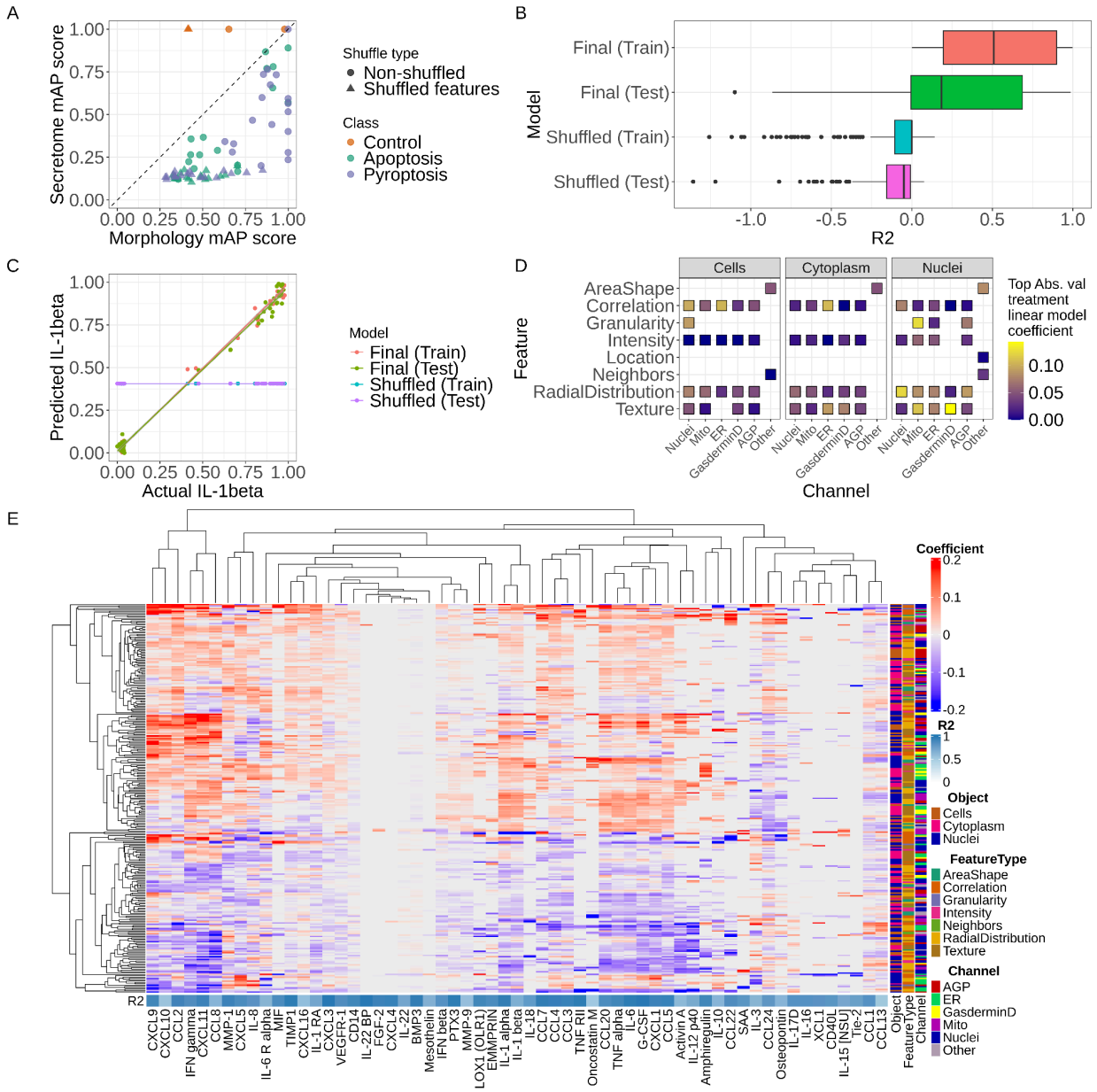

Figure 4. *Cell morphology predicts secretome markers.* **(A)** The mean Average Precision (mAP) of each cell death class across treatment replicates for morphology and secretome. **(B)** The R^2 score for every model (187) across data splits and data shuffles. **(C)** The predicted value of IL-1β compared to the actual value across train and test splits, as well as shuffled baseline and non-shuffled models (see methods), show comparatively high predictive performance. **(D)** The top absolute value logistic regression coefficient for each feature type, compartment, and channel reveals that predicting individual secretome markers uses many different kinds of morphology features. **(E)** Heatmap of the logistic regression coefficients of each selected morphology feature for each selected secretome marker model. Each model has its R^2 score labeled, and each feature has its compartment, feature type, and channel annotated. We order the rows and columns by hierarchical clustering using complete linkage distance metric.

Machine learning predicts cell death phenotypes in single-cells

To better understand single-cell differences in pyroptosis and apoptosis, we trained a fully-connected, two-layer Multi-Layer Perceptron (MLP) using single-cell morphology data to predict cell death mechanism. We defined cell death mechanisms using IL-1β and TNF-α cytokine secretion levels, as shown in **Figure 2A**. We used training, validation, testing, and hold-out t data splits (see methods) to evaluate the performance of our model on single-cells and entire treatments that the model had never before seen (**Supplemental Figure 10**). Our models show high precision and recall, indicating generalizable predictions with minimal overfitting specifically for predicting pyroptosis and control (**Fig. 5A**). We see a much lower predictive performance for apoptosis, likely because of having far fewer single-cells and treatments. Confusion matrices and F1 scores further show particularly high performance for pyroptosis predictions, even in held-out wells (**Fig. 5B**; **Supplemental Figure 11)**. Single-cell class probabilities are skewed toward the correct cell death mechanism, representative single-cell images show differential morphology features that align with canonical understanding by eye (e.g., Gasdermin D translocation to membrane) (**Fig. 5C-E; Supplemental Figure 12**). Importantly, the entire treatment hold-out set performed with an F1 score above 0.75 compared to its shuffled baseline F1 score of less than 0.15 **(Supplemental Figure 13A)**. We show the predicted probability distribution for single-cells in this heldout treatment and a predicted probability of one representative image in **Supplemental Figure 13B-C**.

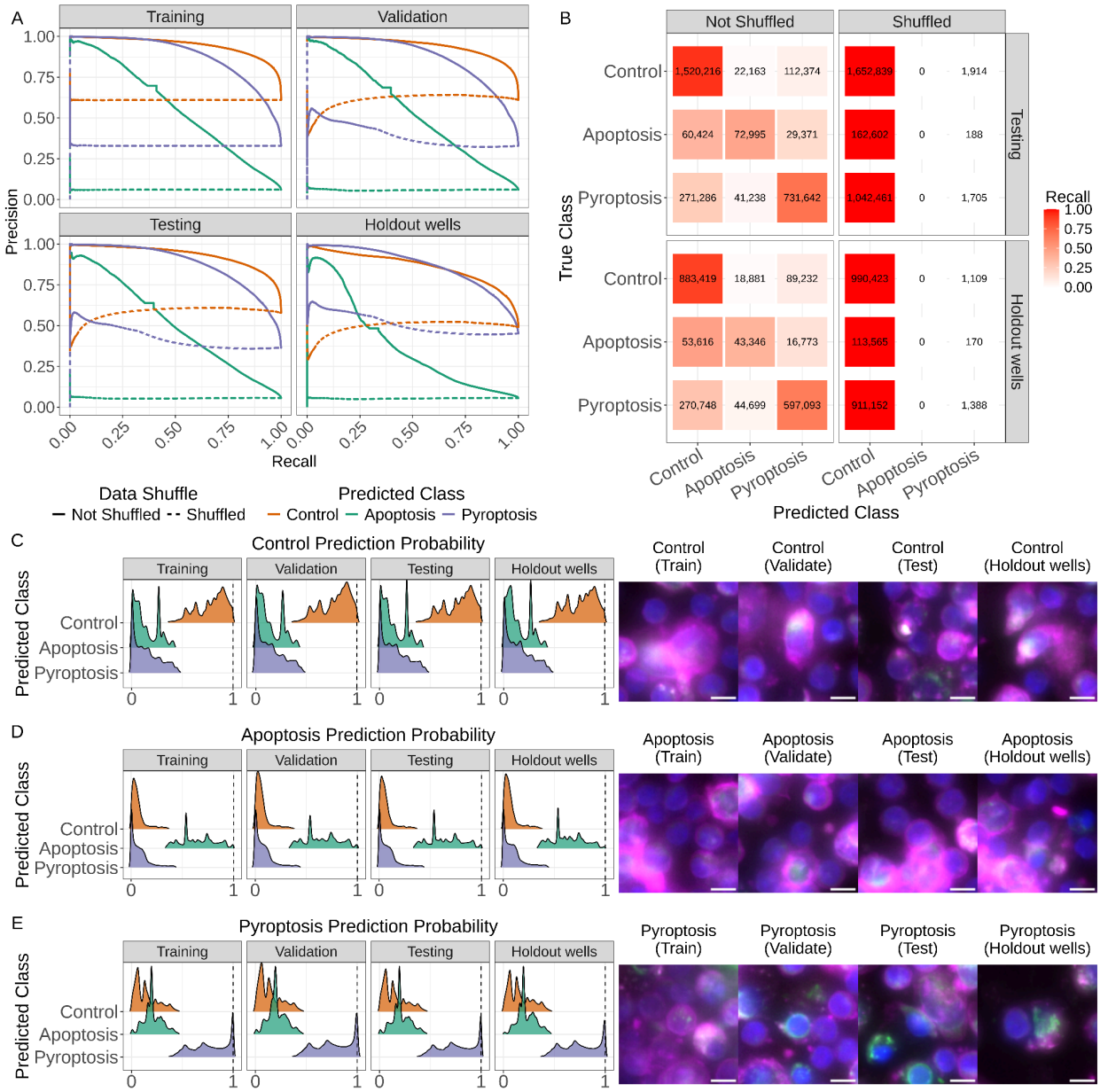

Figure 5. *Machine Learning predicts single-cell death states.* **(A)** Precision-recall curves of the training, validation, testing, and holdout well data splits for both the non-shuffled and shuffled baseline models. **(B)** Confusion matrices for shuffled and non-shuffled models showing the testing and holdout well data splits. The number in each square represents the number of predicted single-cells, and the color represents the recall of each class. Probabilities for **(C)** control, **(D)** apoptosis, and **(E)** pyroptosis predictions stratified by true class. A single-cell cropped image for cells with a probability equal to 1 for each class and data split; where the channels represent: AGP = magenta, Gasdermin D = green, and DNA = blue. AGP is the actin, golgi apparatus, and plasma membrane stain in the canonical Cell Painting panel. The Gasdermin D is the N-terminal cleaved region. Scale bars represent 5μm.

Discussion

In treatment-stimulated PBMCs, paired secretome and high-content imaging demonstrated a complementary map that characterizes pyroptosis. The secretome data confirmed canonical pyroptotic markers (IL-1β and TNF-α), but it also revealed a more dynamic landscape that differs even between pyroptosis treatments. Specifically, the LPS plus nigericin-treated cells showed a different pyroptotic secretome than the flagellin or LPS-treated cells. This could result from the differential activation of TLR4 (LPS),[72] potassium efflux via ionophore channel (nigericin),[78] and/or TLR5 activation (flagellin).[72] We also unexpectedly observed an inflammatory response in cells during apoptosis. Specifically, our apoptotic cells secreted the inflammatory markers IL-2, CCL13, CCL1, CCL24, and osteopontin, which contradicts previous descriptions of the non-inflammatory nature of apoptosis.[79] However, other recent research categorizes these markers as anti-inflammatory and anti-apoptotic[64,70,80,81] or even inflammatory in certain circumstances such as caspase inhibition and mitochondria-initiated apoptosis.[7] Both apoptosis and pyroptosis share similar secretion quantities, implying that many markers are indicative of general cell stress and cell death, or if they are also present in the control group, that they are part of normal PBMC processes (see **Fig S4**).

Our high-content imaging and image-based profiling approach characterized the cell morphology landscape of pyroptosis. Pyroptosis altered nuclei, actin, Golgi, and plasma membrane morphology, while apoptosis had more morphology feature changes, mostly impacting mitochondria. Deep neural networks trained using single-cell morphology features robustly predicted cell death class, even in never-before-seen wells and treatments. Recent approaches have used deep learning to predict cells dying by apoptosis and ferroptosis.[36] The paired secretome data combined with our modified Cell Painting panel to include Gasdermin D, added important layers of ground truth to the imaging data, which enabled us to explore important links between data types. For example, we observed that machine learning models can accurately predict many individual secreted markers using diverse morphology features from different organelles. This suggests a mechanistic link between form and function, but the causal directionality remains unknown (i.e., do single-cell morphology changes indicate preparation for secretome marker secretion, or does secretome marker secretion cause cells to change morphology?). Overall, our map provides a multi-modal, systems biology characterization of pyroptosis, which reveals a complex secretome and morphological landscape that overlaps and diverges from apoptosis.[36] Given that pyroptosis is extensively less studied compared to pyroptosis **(Supplemental Figure 14)**, our map of pyroptosis will provide two major advances for drug discovery and cell death biology: a) enables further mechanistic studies to build on organelle-defined image-based profiles, and b) objective elucidation of key differences between apoptosis and pyroptosis.

There are many limitations to our approach. First, we collected data only six hours after treatment induction, which provided only a snapshot of the early stages of pyroptosis. A cell experiencing pyroptosis might commit to cell death or upregulate proliferative pathways that rescue.[82,83] Nevertheless, a six hour incubation point distinctly separated pyroptosis from apoptosis in both the secretome and cell morphology. We aim to further elucidate the intracellular dynamics of cells undergoing forms of cell death, such as pyroptosis and apoptosis, in a temporal-dependent manner in future studies. Second, we also measured only 187 secretome markers and thus missed the full secretome response. However, with technical advances, 187 markers are more secretome markers than previously imaginable via ELISA-based assays, and we expect content to increase in the future. Cell secretion can be variable and pleiotropic, with multiple secreted secretome markers being indicative of multiple biological processes. Thus we were cautious of oversimplified interpretations due to the

multifaceted nature of cell secretion profiles. Third, the secretome marker profiling captures populations of single cells. Therefore, we are not able to distinguish which cells are secreting secretome markers, and it is possible that certain cells are secreting a disproportionate quantity of secretome markers. However, despite this potential heterogeneity, we still see high performance in using well-aggregated cell morphology to predict many individual secretome markers. Fourth, although a well might have evidence of pyroptotic inflammatory response, every cell might not necessarily be undergoing pyroptosis. This could be a source of false negative classifications of single cells in our deep learning analysis. Fifth, the Cell Painting assay used five imaging channels, and including the Gasdermin D stain introduced some spectral overlap. We combat this issue via feature selection methods that remove highly correlated features across channels (see methods). Nevertheless, our machine learning and comparative analyses identify differential features across all imaging channels. Additionally, we maximally project our images in the Z dimension thus introducing a false positive signal of mitochondria and Gasdermin D stains to the nuclear compartment. And finally, sixth, we are using a relatively small dataset. We treated PBMCs from a single donor with 36 treatment combinations in 154 individual wells. While this yielded approximately 8.3 million segmented single cells, aggregating information at the well level reduced our dataset to 154 well samples. Despite this reduction, we were still able to predict certain secretome markers from morphology reliably and reveal important morphology indicators that were different across cell death classes. It is likely that donor-to-donor differences exist and different cell types could reveal a different pyroptosis map. We expect future studies will expand and refine this pyroptosis map by exploring additional cell lines, resolutions, and adding temporal content.

Materials and Methods

Cell culture

We cultured PBMCs in a controlled environment of 37ºC with 5% CO_2. We plated PBMCs in a consistent medium at an approximate density of 125,000 cells per well (see **Supplemental File 1** for specific conditions). We incubated PBMCs for 30 minutes prior to compound treatment. We also applied a similar cell culture, data collection, and experimental strategy to SH-SY5Y cells, although we do not report these results in this manuscript.

Cell Painting

We modified the canonical Cell Painting assay to remove the cytoplasmic RNA and nucleoli stain for a stain that specifically marks cleaved N-terminal Gasdermin D (Anti-cleaved N-terminal GSDMD Rabbit Monoclonal, Abcam).[27] Additional Cell Painting organelles and stains included Nuclei (Hoechst 33342); the endoplasmic reticulum (Concanavalin A PhenoVue Fluor 488); Golgi apparatus and the plasma membrane (WGA PhenoVue Fluor 555); actin (Phalloidin PhenoVue Fluor 568); and mitochondria (PhenoVue 641 Mitochondria stain).

We followed the version 3 Cell Painting protocol.[84] Briefly, at 6 hours post compound incubation, we treated the cells with live cell mitochondria stain for 30 min at 37°C, 5% CO_2. We then fixed cells in 30 µL of 5.33% (w/v) - final concentration 4% (w/v) of methanol-free PFA in PBS for 20 minutes at room temperature. We then permeabilize in a permeabilization buffer for 5 minutes. Following permeabilization, we added 50 uL of blocking solution for 1 hour at room temperature. We stained cells with Gasdermin D primary antibody overnight at 8°C. We then stained cells with secondary antibody and Hoechst for 1 hour at room temperature. We performed image acquisition prior to completing Cell Painting. We incubated cells in permeabilization buffer and

Cell Painting dyes for 30 minutes at room temperature. The cells were subsequently imaged with all channels **(Supplemental File 1)**.

Image acquisition

We collected microscopy images using the Revvity (previously known as PerkinElmer) Operetta CLS imaging platform with a 20x water immersion NA 1.0 objective with two separate acquisitions. Our first acquisition was prior to the addition of Cell Painting stains. We acquired two channels (Hoechst 33342 and Gasdermin D) at 16 different fields of view (FOV) per well over 3 z-planes spaced out 2µm apart. After Cell Painting stains were added, we acquired images over five channels at 16 different fields of view (FOV) per well at four different z-slices spaced out 2µm apart. Channel excitation and emission wavelengths can be found in **Supplemental Table 4**. We binned pixels at 1x1. We output all Cell Painting in 16-bit TIFF format, preserving data integrity through lossless compression.

Image analysis

We used CellProfiler[73] for image analysis. We applied maximum projection, which takes the highest pixel intensity value per pixel position from all four z-slices. Once there was one z-stack per FOV, we corrected the images for illumination errors, such as vignetting, using the standard CellProfiler approach.[85] We created one illumination correction function per channel and saved these functions as NPY files. We applied these functions to images during the feature extraction pipeline.

Using CellProfiler, we segmented three compartments (nuclei, cells, and cytoplasm) for every single cell. CellProfiler creates binary masks for each compartment per FOV, which are then applied to each of the five channels. We extracted features from each compartment, such as granularity, texture, intensity, and more. We stored feature data in a SQLite database file for downstream image-based profiling. See https://github.com/WayScience/pyroptosis_signature_image_profiling for access to our CellProfiler pipelines.

Image-based profiling

We formatted the extracted features from CellProfiler by performing image-based profiling via the Cytomining software ecosystem **(Supplemental Figure 5)**. We used software called CytoTable[74] to process and clean the CellProfiler SQLite output file. CytoTable merges SQLite compartment tables to create one row per single-cell and outputs the data into the high-performance Apache Parquet format.[86] We then used pycytominer software to perform the rest of the image-based profiling pipeline.[75] The pipeline consists of first annotating the single-cells with metadata from a plate map file associated with each single-cell (e.g., treatment, dose, etc.). We then normalized the features using the per-cell type negative controls (DMSO-treated cells) as a reference using the standardized method (z-score normalization). We performed feature selection on the normalized data to remove redundant or non-informative features. Feature selection consisted of removing features that have a Pearson correlation coefficient of 0.9 or higher, removing blocklist features[87], features that had a low (0.1 ratio) number of unique measurements compared to samples, and features that had a composition of missing values (NA) above 5%. In summary, we segmented and measured 8.3 million single cells and extracted a total of 2,907 features per single-cell (we retained 1,199 features after feature selection).

nELISA panel

Following the Cell Painting data acquisition, we isolated the supernatant from the same treatment wells, which were sent to Nomic Bio (Montreal, Canada) for nELISA-based secretome marker analysis, as described previously.[37] Briefly, the nELISA pre-assembles antibody pairs on spectrally encoded microparticles, resulting in spatial separation between non-cognate antibodies, preventing the rise of reagent-driven cross-reactivity, and enabling multiplexing of hundreds of ELISAs in parallel. The nELISA assay measured 187 secretome markers (see **Supplemental Table S2**), resulting in paired Cell Painting and secretome profiling data at the well level. We processed the nELISA signals using min-max scaling.

Ground truth gating via canonical cytokines

We applied a double gate against IL-1β (0.5) and TNF-α (0.5) to identify wells that included pyroptotic cell death.[39,54–58] We deemed double positive wells to be pyroptotic and double negative wells to be non-pyroptotic. Further, we applied a double gate for IL-1β (0.4) and CCL24 (0.5) to assign apoptotic cell death.[39,81] We considered CCL24 positive and IL-1β negative wells as apoptotic. We considered all wells that were negative for both gates as controls. These processes aligned with our cell death expectations given the existing literature about all compounds, with the exception of the low-dosage flagellin. Combined with a six-hour time point, we anticipate that the low dose of flagellin likely didn't have enough strength to induce apoptosis.

Shuffled baseline models

Across all approaches we utilize in this work, we generate shuffled baseline models in the same way. We randomly permute each sample at the single-cell or single-well level (depending on the data resolution) over every feature in our feature selected data. This approach allows us to test our models on shuffled data while preserving the distribution of the data across each feature.

ElasticNet regression models framework

We trained and evaluated ElasticNet logistic regression models using sklearn v1.3.0.[88,89] We used mean-aggregated well morphology data to predict 187 secretome markers from 1,199 feature-selected morphology features as inputs (normalized data before feature selection). We trained individual models per secretome marker. Further, we trained shuffled baseline models to use as negative controls. To avoid overfitting, we split our data stratified by treatment.
We split 25% of the lowest and highest LPS doses (0.010 ug/mL and 100ug/mL) to the training set and 75% to the testing set. We kept LPS at 1 ug/mL, both with and without Nigericin at 3uM, 100% in the testing set. In addition, we kept all doses of flagellin in the testing set. For all remaining treatments, we performed a 50% training and 50% testing split. We trained the models using a leave-one-out cross-validation (LOOCV) approach and optimized the following model parameters from the following search spaces. We optimized α parameters [0.001, 0.01, 0.1, 1, 10, 100, 1000, 10000] and the L1/L2 ratio [0.01, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.9, 0.99] where L1 = 0 and L2 = 1. We also trained and tested on a permuted feature space to establish a shuffled baseline.

Multi-Layer Perceptron models framework

We designed and trained a multi-class, two-layer, Multi-Layer Perceptron (MLP) to predict cell death types.[90] We used the Pytorch v2.0.0 library for the model creation, training, and testing.[91] We used Optuna v3.3.0[92] to optimize model hyperparameters from the search space described

in **Supplemental Table S5**, which also provides the optimized architecture and hyperparameters. We also trained and tested on a permuted feature space to establish a shuffled baseline.

Representative image selection

We selected a representative image using a predefined metric for all image montages. In this study, we used the predicted probability of a cell's predicted cell death class as the metric of choice to represent correctly predicted cells from our machine learning model **(see Fig. 5)**.

Morphology feature space ANOVA

To determine differential morphology features across control, apoptotic, and pyroptotic groups, we performed an ANOVA (Analysis Of Variances). We fit out data to each morphology feature, with the variate being the cell death groups defined in **Figure 2A** and a covariate of the number of single-cells in the well of the single-cell's origin. We further performed a Tukey's Honestly Significant Difference [HSD] test to adjust p-values and determine group differences.[93]

UMAP visualization

We used UMAP to visualize the dimension reduction of morphology and secretome features.[59] We generated all UMAP plots with six nearest neighbors, a minimum distance of 0.8, and 2 components. We used the cosine similarity metric with a spread of 1.1 and random initialization. To provide better visualization for single-cell morphology, we randomly downsampled to 100 cells per treatment.

Mean Average Precision (mAP) analysis

The Mean Average Precision (mAP) is a metric of how similar a given group is to a control group. We used mAP to evaluate 1) the consistency of cell death categories, 2) the consistency of individual treatments of each cell death category, and 3) the consistency of individual treatments for both secretome and morphology profiles. We also applied mAP to data after applying procedures to shuffle features. We calculated the mAP using the Copairs Python package.[77]

Computational resources

We utilized the Alpine high-performance computing resource at the University of Colorado Boulder. Alpine has 382 compute nodes with 22,180 cores, 36 NVIDIA a100 GPUs, and 24 AMD MI100 GPUs. In addition to Alpine, all other computation was performed on a local workstation with the following specifications: AMD Ryzen 9 5900x CPU - 12 cores, with 128 GB of DDR4 RAM, and GeForce RTX 3090 TI GPU.

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Conflicts of interest

MM, JKa, JKn, and RS are all employees of Assay.Works. JKn and RS are both founders of Assay.Works. MD is both an employee and founder of Nomic Bio. JH, GO, and NR, are all employees of Nomic Bio. The authors declare no other conflicts of interest.

Data and Code Availability

All data and code are publicly available. The raw images, illumination correction files, and extracted feature profiles were deposited to the Image Data Resource (IDR) (https://idr.openmicroscopy.org) under accession number idr0160.

All image analysis pipelines for segmentation, feature extraction, and image-based profiling are available at https://github.com/WayScience/pyroptosis_signature_image_profiling.[94]
All data analysis code is available at:
https://github.com/WayScience/pyroptosis_signature_data_analysis.[95]

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