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**Figure 1. Optical metabolic imaging of primary human B cells activated with IL-4 and anti-CD40 antibody.** (A) B cells were isolated from human peripheral blood and activated for 72 hours with 5 µg/mL anti-CD40 antibody and 20 ng/mL IL-4, or cultured unstimulated. (B) IL-6 concentration was measured in media collected from B cells isolated from two different donors and cultured with or without anti-CD40/IL-4 for 72 hours. The increase in IL-6 concentration in the activated B cell condition is consistent with T-dependent B cell activation. (C) Samples of media from activated and quiescent B cells were taken before imaging and measured using commercial kits. Glucose in the media of activated B cells was significantly decreased compared to the quiescent cell media. (D) Lactate levels in activated B cell media were significantly higher than lactate levels in the quiescent cell media. (E) Representative images of NAD(P)H τm, FAD τm, redox ratio, and anti-CD69 staining in the unstimulated and activated conditions. (F) Redox ratio (NAD(P)H intensity divided by the sum of NAD(P)H and FAD intensity) normalized to the mean of the control group significantly increased in the CD69+ B cells in the IL-4 + anti-CD40 condition compared to CD69- B cells in the unstimulated condition. (G) – (H) NAD(P)H τm significantly decreased and NAD(P)H α1 significantly increased in the CD69+ B cells in the IL-4 + anti-CD40 condition compared to CD69- B cells in the unstimulated condition. (I) A significant decrease in FAD τm was seen in the CD69+ B cells in the IL-4 + anti-CD40 condition compared to CD69- B cells in the unstimulated condition. In (C) – (D), media samples were diluted 100-fold and 0.5μL was assayed. Assays were performed according to the respective BioVision kit protocols. \* P < 0.05, \*\*\*\* P < 0.0001, parametric T-test. In (F) – (I), data are displayed as box-and-whisker plots, representing the median and interquartile range (IQR), with whiskers at 1.5\*IQR. Plots are overlaid with data points; each point represents one cell. n = 1210 cells (461 cells in the activated CD69+ condition, 749 cells in the control CD69- condition). \*\*\*\* P < 0.001, two-tailed unpaired T-test.

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**Figure 2. Heterogeneity and classification of activated and quiescent B cells using OMI parameters.** (A)Heatmap of single-cell data across all B cell experiments. Hierarchical cell clustering was calculated based on the z-scores (the difference between cell mean and population mean divided by the population standard deviation) of ten OMI variables (NAD(P)H τm, τ1, τ2, α1; FAD τm, τ1, τ2, α1; control-normalized optical redox ratio, and cell size as number of pixels). The single-cell clustering demonstrates that using all OMI variables, activated B cells tend to group separately from quiescent B cells regardless of donor. (B) UMAP of ten OMI parameters visualizes separation between clusters of activated (CD69+ in activated condition) and quiescent (CD69- in unstimulated condition) B cells. (C) Pie chart showing the relative weight of the ten OMI variables included in the “all variables” random forest classifier. (D) Receiver operating characteristic (ROC) curve of random forest classifiers trained for classification of quiescent and activated B cells on different combinations of OMI variables, with operating points indicated. “Top variables” classifiers refer to the largest weighted variables in the “all variable” classifier, found in (C). An area under the curve (AUC) of 0.99 is indicative of high performance of the “all variable” classifier and the NAD(P)H variables (NAD(P)H τm, τ1, τ2, α1) + cell size classifier. n = 1210 cells (461 cells in the activated CD69+ condition, 749 cells in the control CD69- condition) with a 70/30 split for training and test sets.

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**Figure 3. Optical metabolic imaging of primary human NK cells activated with IL-12, IL-15, and IL-18.** (A) NK cells were isolated from human peripheral blood and activated with 10 ng/mL IL-12, 50 ng/mL IL-15, and 50 ng/mL IL-18 for 24 hours. (B) IFN-y concentration in media collected from NK-cells isolated from two different donors and cultured with or without activating cytokines for 24 hours. The increase of IFN-y in the activated condition is consistent with NK cell activation. C) Samples of media from activated and quiescent NK cells were taken before imaging and measured using commercial kits. Glucose in the media of activated NK cells was significantly decreased compared to the quiescent cell media. (D) Lactate levels in activated NK cell media were significantly higher than lactate levels in the quiescent cell media. (E) Representative images of NAD(P)H τm, FAD τm, redox ratio, and anti-CD69 staining in the control and activated conditions. (F) Redox ratio significantly increased in the CD69+ NK cells in the cytokine-activated condition compared to CD69- NK cells in the unstimulated condition. (G) – (H) NAD(P)H τm significantly decreased and NAD(P)H α1 significantly increased in the CD69+ NK cells in the cytokine-activated condition compared to CD69- NK cells in the unstimulated condition. (I) FAD τm did not significantly change between the CD69+ NK cells in the cytokine-activated condition compared to CD69- NK cells in the unstimulated condition. In (C) – (D), media samples were diluted 100-fold and 0.5μL was assayed. Assays were performed according to the respective BioVision kit protocols. \*\*\*\* P < 0.0001, parametric T-test. In (F) – (I), data are displayed as box-and-whisker plots, representing the median and interquartile range (IQR), with whiskers at 1.5\*IQR. Plots are overlaid with data points; each point represents one cell. n = 1702 cells (552 cells in the activated CD69+ condition, 1150 cells in the control CD69- condition). \*\*\*\* P < 0.001, two-tailed unpaired T-test.

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**Figure 4. Heterogeneity and classification of activated and quiescent NK cells using OMI parameters.** (A) Heatmap of single-cell data across all NK cell experiments reveals heterogeneity within the dataset. Hierarchical cell clustering was calculated on the z-scores (the difference between cell mean and population mean divided by the population standard deviation) of ten OMI variables (NAD(P)H τm, τ1, τ2, α1; FAD τm, τ1, τ2, α1; control-normalized optical redox ratio, and cell size as number of pixels) and revealed that cells from Donor E generally clustered away from Donors D and F. Additionally, two separate clusters of CD69+ cells formed within the donor-level clusters. (B) UMAP of ten OMI parameters displays clustering of activated (CD69+ in activated condition) and quiescent (CD69- in unstimulated condition) NK cells. Two distinct clusters containing both activated and quiescent cells are visualized in the UMAP. (C) Pie chart showing the relative weight of each of the ten OMI parameters in the “all variable” random forest classifier. (D) ROC curve of random forest classifiers trained for classification of quiescent and activated NK cells based on different combinations of OMI parameters, with operating points indicated. “Top variables” classifiers refer to the largest weighted OMI parameters in the classifier using all variables, displayed in (C). The classifier using all 10 OMI parameters performed the best (AUC 0.91), followed by the classifier that used NAD(P)H lifetime variables (NAD(P)H τm, τ1, τ2, α1) and cell size (AUC 0.88).n = 1702 cells (552 cells in the activated CD69+ condition, 1150 cells in the control CD69- condition) with a 70/30 split for training and test sets.

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**Figure 5. Classification of lymphocyte activation status based on OMI parameters collected in B cells, NK cells, and previously published T-cell data.** Data from activated and quiescent T-cells (published in Walsh et. al. 2021), B cells, and NK cells was used to evaluate OMI measurements across lymphocytes. (A) Box-and-whisker plots of key OMI variables (control-normalized optical redox ratio, NAD(P)H α1, and NAD(P)H τm) display consistent changes with activation across T-cells, B cells, and NK cells. Additionally, NAD(P)H τm and NAD(P)H α1values differ between quiescent (CD69- control) cells in each of the three lymphocyte subtypes (comparisons between quiescent groups were not computed for the optical redox ratio, due to normalization). (B) Heatmap displaying hierarchical clustering of groups of activated or quiescent cells by lymphocyte subtype, donor, and activation status, calculated from the z-scores (the difference between experimental group mean and the mean of all cells divided by the standard deviation of all cells) of ten OMI variables. (C) UMAP of single-cell OMI data displays distinct clusters of lymphocytes based on lymphocyte subtype and activation status. (D) ROC curves of random forest classifiers trained to identify activated cells across all three lymphocyte subtypes, with operating points indicated. The highest weighted OMI parameters were used in the “top variables” classifiers; these weights are in Supp. Fig. 5C. (E) Accuracy of different random forest classifiers trained to identify lymphocyte subtype (one vs one approach). Variable weights for "top variables” are in Supp. Fig. 6B. (F) Accuracy of random forest classifiers trained to identify lymphocyte subtype and activation across all three lymphocyte subtypes (one vs. one approach) using different OMI parameters. Variable weights are in Supp. Fig. 8B. n = 7739 cells (749 CD69- control B cells, 461 CD69+ activated B cells, 1150 CD69- control NK cells, 552 CD69+ activated NK cells, 3234 CD69- control T-cells, 1593 CD69+ activated T-cells) with a 70/30 split for training and test sets. \*\*\*\* P < 0.001, ANOVA + Tukey HSD.

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**Supplemental Figure 1. OMI of B CD69+ and CD69- B cells in both control and anti-CD40 + IL-4 activated culture.** Both CD69+ and CD69- B cells in both conditions (control and IL-4 + αCD40 activated) for each OMI parameter: (A) optical redox ratio (NAD(P)H intensity divided by the sum of NAD(P)H + FAD intensities), (B) NAD(P)H mean lifetime τm, (C) FAD mean lifetime τm, (D) unbound NAD(P)H fraction α1, (E) unbound NAD(P)H lifetime τ1, (F) protein-bound NAD(P)H lifetime τ2, (G) protein-bound FAD fraction α1, (H) protein-bound FAD lifetime τ1, (I) unbound FAD lifetime τ2, (J) cell size (defined as number of pixels in cell). Box-and-whisker plots display single cell values (dots) overlaid on box-and whisker plots displaying the median, interquartile range (IQR), and minimum/maximum value. n = 1352 (461 cells in the activated CD69+ condition, 130 cells in the activated CD69- condition, 12 cells in the control CD69+ condition, 749 cells in the control CD69- condition). \* p < 0.05, \*\*\* p < 0.005, \*\*\*\* p < 0.001, ANOVA + Tukey’s HSD.

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**Supplemental Figure 2. Additional UMAPs and classifier performance for single B cell OMI.** (A) UMAP of B cells with labels for both CD69+ and CD69- cells in the control and activated (anti-CD40+IL4) groups. (B) UMAP of B-cells color-coded by donor (A, B, C) and activation status (CD69+, CD69-). (C) Confusion matrix of the 10 OMI parameter random forest classifier shows similar performance for classification of CD69+ activated and CD69- control B cells (True positive rate = 0.92, false positive rate = 0.04, accuracy = 94.2%). (D) Confusion matrix of a logistic regression classifier trained on 10 OMI parameters to classify B cells as CD69+ activated or CD69- control (True positive rate = 0.93, false positive rate = 0.06, accuracy = 93.4%). (E) Confusion matrix of a support vector machine (SVM) classifier trained on 10 OMI parameters to classify B cells as CD69+ activated or CD69- control (True positive rate = 0.90, false positive rate = 0.06, accuracy = 92.3%). (F) ROC curves for random forest, logistic regression, and SVM classifiers trained on 10 OMI parameters, with operating points indicated. The random forest classifier performed the best, with an AUC of 0.99. In (A), n = 1352 (461 cells in the activated CD69+ condition, 130 cells in the activated CD69- condition, 12 cells in the control CD69+ condition, 749 cells in the control CD69- condition). In (B) – (F), n = 1210 cells (461 cells in the activated CD69+ condition, 749 cells in the control CD69- condition) with a 70/30 split for training and test sets.

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**Supplemental Figure 3. OMI of CD69+ and CD69- NK cells in both unstimulated and IL-12 + IL-15 + IL-18 activated culture conditions.** Both CD69+ and CD69- NK cells in both conditions (control and IL-12 + IL-15 + IL-18 activated) for each OMI parameter: (A) optical redox ratio (NAD(P)H intensity divided by the sum of NAD(P)H + FAD intensities), (B) NAD(P)H mean lifetime τm, (C) FAD mean lifetime τm, (D) unbound NAD(P)H fraction α1, (E) unbound NAD(P)H lifetime τ1, (F) protein-bound NAD(P)H lifetime τ2, (G) protein-bound FAD fraction α1, (H) protein-bound FAD lifetime τ1, (I) unbound FAD lifetime τ2, (J) cell size (defined as number of pixels in cell). Box-and-whisker plots display single cell values (dots) overlaid on box-and whisker plots displaying the median, interquartile range (IQR), and minimum/maximum value. n = 2699 cells (552 activated CD69+ cells, 691 activated CD69- cells, 306 control CD69+ cells, 1150 control CD69- cells). \* p < 0.05, \*\* p < 0.01, \*\*\*\* p < 0.001, ANOVA + Tukey’s HSD.

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**Supplemental Figure 4. Additional UMAPs and classifier performance for single NK cell OMI.** (A) UMAP of NK cells with labels for both CD69+ and CD69- cells in the control and activated (IL-12 + IL-15 + IL-18) groups. (B) UMAP of NK cells color-coded by donor (D, E, F) and activation status (CD69+, CD69-). (C) Confusion matrix of the 10 OMI parameter random forest classifier trained to classify NK cells as CD69+ activated or CD69- control cells (True positive rate = 0.68, false positive rate = 0.08, accuracy = 84.3%). (D) Confusion matrix of a logistic regression classifier trained on 10 OMI parameters to classify NK cells as CD69+ activated or CD69- control (True positive rate = 0.59, false positive rate = 0.11, accuracy = 79.3%). (E) Confusion matrix of SVM classifier trained on 10 OMI parameters to classify NK cells as CD69+ activated or CD69- control (True positive rate = 0.65, false positive rate = 0.12, accuracy = 80.4%). (F) ROC curves for the random forest, logistic regression, and SVM classifiers trained on 10 OMI parameters, with operating points indicated. The random forest classifier performed the best, with an AUC of 0.91. In (A), n = 2699 cells (552 activated CD69+ cells, 691 activated CD69- cells, 306 control CD69+ cells, 1150 control CD69- cells). In (B) – (F), n = 1702 cells (552 activated CD69+ cells, 1150 control CD69- cells) with a 70/30 split for training and test sets.

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**Supplemental Figure 5. Additional UMAPs and classifier performance for activation of lymphocytes (T cells, B cells and NK cells).** (A) UMAP of single-cell OMI data from Fig. 5C containing all T, B, and NK cells color-coded by activation status. (B) Bar graph of % accuracy for a random forest classifier trained to distinguish CD69+ from CD69- cells across the combined dataset of all lymphocyte subtypes. (C) Pie chart displaying the weights of OMI variables included in the random forest classifier using all 10 OMI features. (D) ROC curves of random forest, logistic regression, and support vector matrix (SVM) classifiers using all 10 OMI variables to distinguish CD69+ from CD69- cells across all lymphocyte subtypes, with operating points indicated. (E) Confusion matrix for random forest classifier using all 10 OMI variables demonstrates similar performance in correctly classifying cells as activated (CD69+) or quiescent (CD69-) (True positive rate = 0.87, false positive rate = 0.03 , accuracy score = 93.4%). (F) Confusion matrix for logistic regression classifier (True positive rate = 0.73, false positive rate = 0.05, accuracy score = 87.4%). (G) Confusion matrix for SVM classifier (True positive rate = 0.73, false positive rate = 0.06, accuracy score = 86.3%). n = 7739 cells (5133 CD69- cells, 2606 CD69+ cells) with a 70/30 split for training and test sets.

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**Supplemental Figure 7. UMAP and classifier performance for lymphocyte subtype classifier based on quiescent cells only (T cells, B cells and NK cells).** (A) UMAP of lymphocytes from quiescent (CD69- control) NK, B, and T cells color-coded by lymphocyte subtype. (B) Bar graph displaying accuracy of random forest classifiers trained to separate lymphocytes based on lymphocyte subtype (one vs. one approach, quiescent cells only). The classifier with all 10 OMI parameters performed the best (accuracy = 99.5%). Other classifiers with strong performance included: 9 OMI parameters (redox excluded) (accuracy = 99.5%), NAD(P)H lifetime variables + cell size (97.8%), and top 4 OMI parameters (NADH α1, NADH τm, FAD τ1, FAD τm) (accuracy = 97.3%). (C) Feature weights of 10 OMI parameters used for one-vs.-one random forest classification by lymphocyte subtype in (B). (D) Confusion matrix for 10 OMI parameter random forest classifier in (B) (accuracy = 99.5%). n = 5133 cells (749 B cells, 1150 NK cells, 3234 T cells) with a 70/30 split for training and test sets.

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**Supplemental Figure 8. Additional UMAPs and classifier performance for both lymphocyte subtype (T cells, B cells and NK cells) and activation.** (A) UMAP of lymphocytes from Fig. 5C color-coded by lymphocyte subtype, activation status, and donor. (B) Feature weights of 10 OMI parameters used for one-vs.-one random forest classification by lymphocyte subtype and activation status in Fig. 5F (C) Confusion matrix for 10 OMI parameter random forest classifier in Fig. 5F (accuracy = 92.9%). The confusion matrix shows that most confusion between classes occurs between activated or quiescent cells within a lymphocyte subtype. n = 7739 cells (749 CD69- control B cells, 461 CD69+ activated B cells, 1150 CD69- control NK cells, 552 CD69+ activated NK cells, 3234 CD69- control T-cells, 1593 CD69+ activated T-cells) with a 70/30 split for training and test sets.

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**Supplemental Figure 9. Phasor-based classification of NK cell and B cell activation and lymphocyte subtype.** (A) NAD(P)H phasor plot of B cells from Fig. 1 (Red = activated CD69+ B cells, blue = quiescent CD69- B cells). (B) ROC curve and confusion matrix for logistic regression classification of B cell activation. The NAD(P)H phasor at the laser repetition frequency (80MHz) predicted B cell activation with true positive rate = 0.92, false positive rate = 0.06, accuracy score = 93.2% (ROC AUC = 0.953). n = 1355 B cells (469 cells in the activated CD69+ condition, 886 cells in the control CD69- condition) with a 50/50 split for training and test sets (C) NAD(P)H phasor plot of NK cells from Fig. 3 (Red = activated CD69+ NK cells, blue = quiescent CD69- NK cells). (D) ROC curve and confusion matrix for logistic regression classification of NK cell activation. The NAD(P)H phasor at the laser repetition frequency (80MHz) predicted NK cell activation with true positive rate = 0.76, false positive rate = 0.09, accuracy score = 85.7% (ROC AUC = 0.906). n = 1697 cells (547 cells in the activated CD69+ condition, 1150 cells in the control CD69- condition) with a 50/50 split for training and test sets (E) Phasor plots of NAD(P)H (top) and FAD (bottom) of B cells and NK cells at both the laser repetition rate (80 MHz) and its second harmonic (160 MHz) (Red = NK cells, blue = B cells). (F) ROC curve and confusion matrix for logistic regression classification of lymphocyte subtype (B and NK cells). Using both the NAD(P)H and FAD phasors at 80MHz and 160MHz, the logistic regression model could classify a cell as B or NK with true positive rate = 0.98, false positive rate = 0.002, accuracy score = 99.1% (ROC AUC = 0.995). n = 3052 cells (1355 B cells, 1697 NK cells) with a 50/50 split for training and test sets.

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**A**

**B**

**C**

**Supplemental Figure 10. Phasor-based classification of NK cell, B cell, T cell lymphocyte subtype and activation.** (A) NAD(P)H phasor plot of B cells (blue), NK cells (red), and T cells (green). (B) Confusion matrix for random forest classification of lymphocyte subtype. The classifier was trained on NAD(P)H phasors (at the laser repetition frequency 80MHz and its second harmonic 160 MHz) and cell size and achieves a classification accuracy of 93.5%. (C) Confusion matrix for random forest classification of lymphocyte subtype and activation trained on NAD(P)H phasors and cell size. The classifier predicted lymphocyte subtype and activation with an accuracy of 81.4%. n = 1355 B cells (469 B cells in the activated CD69+ condition, 886 B cells in the control CD69- condition); n = 1697 NK cells (547 NK cells in the activated CD69+ condition, 1150 NK cells in the control CD69- condition); n = 3659 T cells (905 cells in the activated condition, 2754 cells in the control condition); a 50/50 data split for training and test sets was used.