

Targeted flux analysis through the Mass Isotopomer Multi-Ordinate Spectral Analysis (MIMOSA) Workflow

Using the QTRAP 6500+ LC-MS/MS System with SelexION® Technology and Elucidata Software

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Fluxomics is a relatively recent application of mass spectrometry-based techniques that employs the use of stable labeled isotopes to trace how specific metabolites and molecules are metabolized through biological pathways. Commonly, ¹³C or ¹⁵N labeled precursor molecules are fed into a biological system and allowed to be metabolized for a given amount before the experiment is quenched. The experimental design can include a single time point once the system reaches a steady-state or a series of time points for a kinetic understanding of how the metabolites are changing over time. Unlike isotope labeling experiments, which mainly focus on the incorporation of labeled atoms into certain metabolites, the output of a flux experiment is a rate. A flux rate reveals details about how a biological system changes between 2 or more cohorts. For a metabolite with an increased enrichment of label, a flux rate can distinguish if this is due to the pathway speeding up and forming more product, or accumulation due to subsequent steps in the pathway slowing down. While fluxomics is a powerful technique in understanding the dynamic changes in metabolism, these experiments require



an expert level of understanding of the pathways and are mathematically rigorous.

The Kibbey lab has developed a targeted fluxomics technique, designated M.I.M.O.S.A. (Mass Isotopomer Multi-Ordinate Spectral Analysis), which is focused on energy metabolism.¹ The MIMOSA technique utilizes a targeted MRM-based method to understand each isotopologue as well as the position of the labeled carbon atoms. For separation of the glycolytic and TCA cycle intermediates, the method employs two 5-min LC gradients in conjunction with differential mobility separation. MIMOSA has been fully incorporated into the Polly software (Elucidata) with their LC-MS/MS labeled workflow. This automated workflow has removed the barriers and improved the rigor for fluxomics research.

Key features of MIMOSA fluxomics

- Uses the QTRAP® 6500+ System with SelexION® Technology for high sensitivity and specificity
- Describes fluxes for glycolytic and TCA cycle intermediates for energy metabolism studies
- Deconvolves the labeling patterns at the MS/MS level to measure positional enrichment
- Experimental output calculates normalized rates, pathway directionality, and dynamic metabolic changes between cohorts
- Polly software fully automates the data processing (natural abundance correction, absolute % enrichment, background correction, flux calculations) and data visualization which dramatically reduces the sample-to-graph time

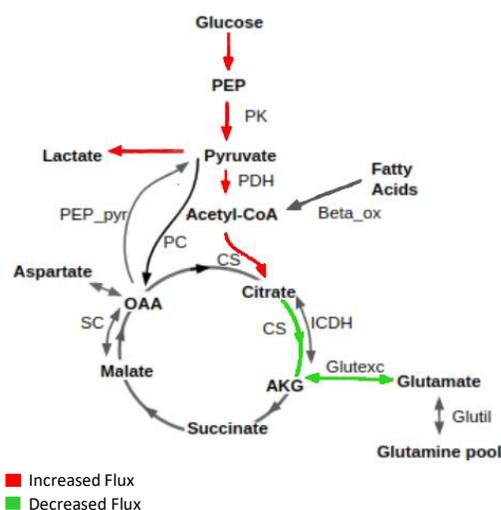


Figure 1. Fluxes were mapped to central carbon pathways to summarize the metabolic changes that occur in response to T-cell activation. The pathway map was generated in the Polly LC-MS/MS labeled workflow (Elucidata). Red indicates increases in flux and green indicates decreases in activated T-cells.

- Polly is flexible and customizable and is capable of handling other stable labeled isotopes

Methods

Sample preparation: A protocol detailing the isolation, culture, and activation of primary human CD4+ T-cells is described in Hiemer *et al.*⁴ Briefly, cells were unstimulated or stimulated with ImmunoCult. Cells were harvested and incubated with fresh cold media without glucose or glutamine (MP Biomedicals) that had been supplemented with sodium bicarbonate (3.7 g/L), 10 mM HEPES, 0.2% essentially FFA free BSA (Sigma), 0.05 mM sodium pyruvate, 4 mM glutamine, and 0.45 mM L-lactate for 1 h. Cells were washed with PBS and resuspended in ¹³C-glucose labeled media that consisted of 5.5 mM [U-¹³C₆]-D-glucose (CLM-2001; Cambridge Isotopes). Cells were incubated in glucose labeling media for 4 h. Cells were subsequently washed with ice-cold PBS, and cellular metabolism was quenched with ice-cold 20% methanol in 0.1% formic acid supplemented with 10 μM d₄-taurine (Cambridge Isotopes), 3 mM sodium fluoride (Sigma), 1 mM phenylalanine (Sigma), and 100 μM EDTA. Cells were frozen in V-bottom plates (Bioexpress, Cat #T-3025-8B) and lyophilized overnight to dryness. Samples were resuspended in water and 2 μL were injected on column.

Chromatography: The M.I.M.O.S.A chromatography has been described in Alves *et al.*¹ In short, glycolytic intermediates were analyzed using an Amino Acid 50 x 2.1 mm, 3 μm column (Imtakt). The TCA cycle intermediates were analyzed using a Hypercarb 100 x 2.1 mm column (ThermoFisher). The gradient conditions for both are described in Table 1.

Mass spectrometry: MS analysis was performed on the QTRAP 6500+ System equipped with SelexION Technology and an IonDrive™ Turbo V Ion Source. The details of the MS

Table 1. Chromatography gradients used.

Glycolytic intermediates		TCA cycle intermediates	
Time (mins)	%B	Time (mins)	%B
Flow rate = 600 μL/min		Flow rate = 400 μL/min	
0	90	0	0
0.75	90	1	0
2	70	1.5	20
3.6	70	3.2	20
4	90	3.3	0
5	90	4	0
Mobile phase A – 100 mM ammonium acetate, pH 6.9 Mobile phase B – acetonitrile		Mobile phase A – 15 mM ammonium formate, 20 μL of 0.5 mM EDTA pH 6.9 Mobile phase B – acetonitrile	

experiments have been described in Alves *et al.*¹ Briefly, the instrument parameters for the glycolytic intermediates experiment were the following: CAD 9, Curtain gas 30, IonSpray Voltage -3000, Temp 450, GS1 60, GS2 60, DMS modifier flow 250 μL/min, DMS temp low, SV 2200, DR off, DMS Modifier 2-propanol, DMS Modifier Composition low, Dwell 10 msec, Pause 3 msec. The instrument parameters used for the TCA cycle intermediates were: CAD 9, Curtain gas 30, IonSpray Voltage -3000, Temp 450, GS1 55, GS2 55, DMS modifier flow 325 μL/min, DMS temp medium, SV 2600, DR open, DMS Modifier 2-propanol, DMS Modifier Composition low, Dwell 10 msec, Pause 3 msec.

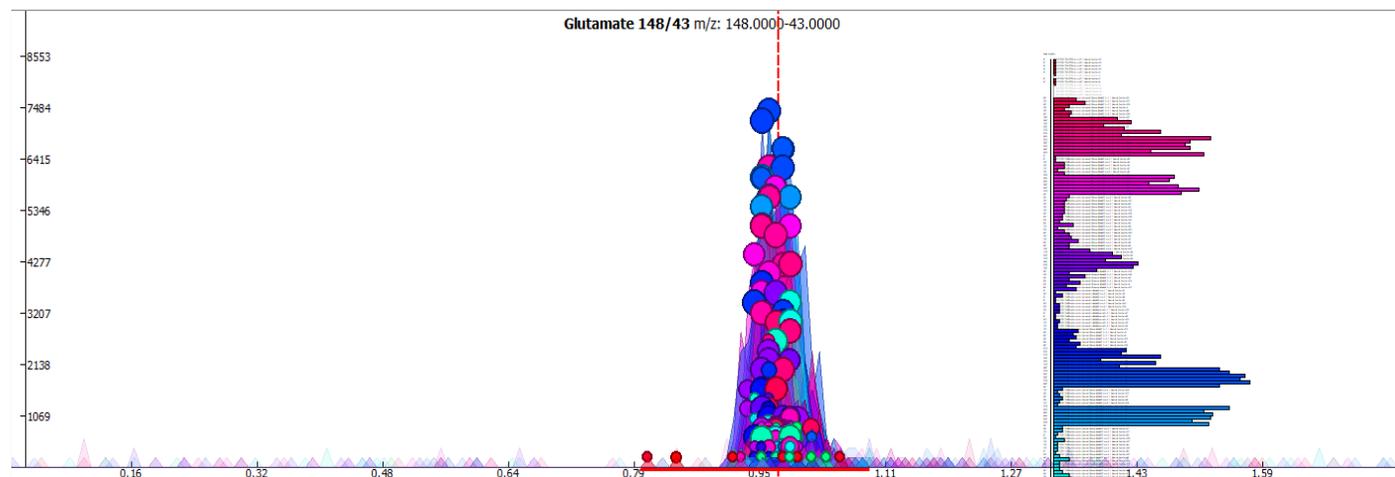
Data processing: The output .wiff data files were converted to mzML using MSConvert (version 3.0.19172, Proteowizard). Peak alignment and integration were performed using EI-Maven v0.12.0. Natural abundance correction, fractional enrichment, background correction, flux calculations, and graphical data visualization were calculated using Polly LC-MS/MS labeled workflow (Elucidata).

Flux analysis on T-cells

T-cells are known to employ a different metabolic program depending upon their activation state.² While in their resting state, T-cells utilize B-oxidation of fatty acids as a major energy source. Once stimulated, T-cells exhibit the Warburg effect and switch to aerobic glycolysis producing increased amounts of lactate. The uptake of glucose, glutamine, and fatty acids from the exogenous environment is required for T-cells to rapidly divide and mount an immune response.³ Here, the steady state metabolic changes that occur in human T-cells 24 hours after activation was studied using a targeted MIMOSA fluxomic approach with SCIEX QTRAP 6500+ System equipped with SelexION Technology and the Polly platform for data processing and visualization.

Data processing using EI-Maven

Flux data acquired on QTRAP 6500+ System with SelexION Technology were converted to mzML to process in EL-Maven for peak alignment and peak integration. Alternatively, MultiQuant™ Software (SCIEX) could have been used for peak integration, then uploaded into Polly as a .csv file with the proper format. In Figure 2 (top), M+2 glutamate (148/43) is used to showcase how the data are visually represented in a single plot with multiple overlaid traces. The bar graph on the right side denotes the peak area for each sample allowing for collective assessment of the data. Viewing the data in this manner allows for fast peak review, instead of the hundreds or thousands of mouse clicks performed when reviewing each scan independently. Importantly, manual integrations of the data can



B

#	ID	Observed m/z	Expected m/z	rt	rt delta	#peaks	#good	Max Width	Max AreaTop	Max S/N	Max Quality	MS2 Score	#MS2 Events	Rank
12	Glutamate 146/41	41.0000	41.0000	0.97	0.00	156	153	24	5.14e+04	905	0.85	0.00	0	2.324764e-02
28	Glutamate 146/128	128.0000	128.0000	1.00	0.03	156	153	28	3.11e+06	259	0.84	0.00	0	1.737465e-02
13	Glutamate 147/41	41.0000	41.0000	0.97	0.00	155	134	14	3.07e+03	440	0.85	0.00	0	3.169159e-02
10	Glutamate 147/42	42.0000	42.0000	0.97	0.00	153	104	13	1.87e+03	260	0.85	0.00	0	3.381147e-02
27	Glutamate 147/129	129.0000	129.0000	1.00	0.03	156	148	28	2.27e+06	1380	0.85	0.00	0	1.739360e-02
9	Glutamate 148/41	41.0000	41.0000	0.97	0.00	149	68	14	3.45e+04	800	0.84	0.00	0	2.449962e-02
8	Glutamate 148/42	42.0000	42.0000	0.96	0.01	142	8	11	1.6e+03	320	0.84	0.00	0	3.553732e-02
7	Glutamate 148/43	43.0000	43.0000	0.97	0.00	149	72	13	5.73e+03	740	0.85	0.00	0	2.912987e-02
26	Glutamate 148/130	130.0000	130.0000	0.98	0.01	155	134	24	1.65e+06	2381	0.85	0.00	0	1.761282e-02
6	Glutamate 149/41	41.0000	41.0000	0.96	0.01	117	9	9	733	140	0.84	0.00	0	4.008596e-02
5	Glutamate 149/42	42.0000	42.0000	0.98	0.01	81	6	12	867	160	0.79	0.00	0	4.619921e-02
4	Glutamate 149/43	43.0000	43.0000	0.98	0.01	147	18	11	1.07e+03	160	0.83	0.00	0	3.836824e-02
25	Glutamate 149/131	131.0000	131.0000	1.26	0.29	150	40	19	2.27e+03	320	0.83	0.00	0	3.480655e-02
24	Glutamate 149/131	131.0000	131.0000	0.97	0.00	153	126	25	7.26e+04	3983	0.85	0.00	0	2.233860e-02
29	Glutamate 150/43	43.0000	43.0000	0.97	0.00	90	19	10	933	160	0.83	0.00	0	3.994110e-02
23	Glutamate 150/132	132.0000	132.0000	0.98	0.01	137	71	21	4.2e+04	2161	0.85	0.00	0	2.351062e-02
22	Glutamate 151/133	133.0000	133.0000	0.98	0.01	132	49	16	1.35e+04	800	0.85	0.00	0	2.672518e-02
57	Glutamine 145/127	127.0000	127.0000	1.04	0.00	156	156	32	1.71e+06	57	0.84	0.00	0	1.804220e-02
63	Glutamine 146/128	128.0000	128.0000	1.00	0.04	156	153	28	3.11e+06	259	0.84	0.00	0	1.737465e-02
18	Glutamine 147/129	129.0000	129.0000	1.00	0.04	156	148	28	2.27e+06	1380	0.85	0.00	0	1.739360e-02

Figure 2. The output from Polly LC-MS/MS labeled workflow after natural abundance correction. Additional tabs include fractional enrichment data, background correction, and a merged table. All data can be easily exported as a .csv file.

be performed as needed. EI-Maven outputs processed data as a table that contains detailed information about MRM transitions, peak area, retention time, S/N, and number of peaks detected as how many are considered good peaks (Figure 2, bottom). This table is used to QC the data where the user can denote a detected metabolite as either good or bad with a checkmark or an X, respectively. The data can be automatically transferred to Polly using EI-Maven's upload to the cloud function which requires an active Polly account.

Polly LC-MS/MS labeled workflow

The required files for workflow include a peak areas file (EI-Maven output), a metadata file detailing the metabolites detected (molecular formula, fragment, isotope label), and a sample metadata file that denotes which cohort each sample belongs to for comparison. After the files are uploaded, the user can access the NA correction tool to perform the natural abundance

correction. Natural abundance correction involves numerous calculations that considers the natural occurrence of the isotope ($^{13}\text{C} = 1.1\%$), the number of carbons in the parent molecular and resultant fragment with all the possible labeling combinations. Setting up these calculations is laborious, time consuming, and the introduction of user error at this step is quite common. In contrast, with this automated workflow, natural abundance is calculated with a single mouse click (Figure 3).

Perform Background Correction
 Replace Negatives with Zero
 PERFORM NA CORRECTION

Table

NA Corrected Values
 Fractional Enrichment
 Background Corrected
 Merged Output

Show 10+ entries Search:

Label	Sample	NA Corrected	Name	Formula	metab_name	Pool_total	Truth Value	NA Corrected with zero
1909 C13_89_0_89_0	20170320_TcellTimeCourse-4uL-Activated Glucose labeledovernight-1 (sample 36)	268900.184854	Lactate 89/89	C3H5O3	Lactate	3273287.894206	false	268900.184854
1910 C13_89_0_89_0	20170320_TcellTimeCourse-4uL-Activated Glucose labeledovernight-2 (sample 49)	266001.69569	Lactate 89/89	C3H5O3	Lactate	4250017.72	false	266001.69569
1911 C13_89_0_89_0	20170320_TcellTimeCourse-4uL-Activated Glucose labeledovernight-3 (sample 96)	257057.822878	Lactate 89/89	C3H5O3	Lactate	3594409.056372	false	257057.822878
1912 C13_89_0_89_0	20170320_TcellTimeCourse-4uL-Activated Glucose labeledovernight-4 (sample 141)	254629.981198	Lactate 89/89	C3H5O3	Lactate	3064944.402846	false	254629.981198
1913 C13_89_0_89_0	20170320_TcellTimeCourse-4uL-Activated Glucose labeledovernight-5 (sample 14)	236281.642099	Lactate 89/89	C3H5O3	Lactate	3008406.482499	false	236281.642099
1914 C13_89_0_89_0	20170320_TcellTimeCourse-4uL-Activated Glucose labeledovernight-6 (sample 120)	192260.096528	Lactate 89/89	C3H5O3	Lactate	3315059.106528	false	192260.096528
1915 C13_89_0_89_0	20170320_TcellTimeCourse-4uL-Activated Glutamine labeled4 hr-1 (sample 135)	4121142.93895	Lactate 89/89	C3H5O3	Lactate	4121142.93895	false	4121142.93895
1916 C13_89_0_89_0	20170320_TcellTimeCourse-4uL-Activated Glutamine labeled4 hr-2 (sample 128)	4562933.9705	Lactate 89/89	C3H5O3	Lactate	4570935.208481	false	4562933.9705
1917 C13_89_0_89_0	20170320_TcellTimeCourse-4uL-Activated Glutamine labeled4 hr-3 (sample 62)	4572185.62205	Lactate 89/89	C3H5O3	Lactate	4599366.345289	false	4572185.62205
1918 C13_89_0_89_0	20170320_TcellTimeCourse-4uL-Activated Glutamine labeled4 hr-4 (sample 116)	5617033.78285	Lactate 89/89	C3H5O3	Lactate	5670505.668695	false	5617033.78285

Showing 1 to 10 of 144 entries (filtered from 2,628 total entries) Previous **1** 2 3 4 5 ... 15 Next

Figure 3. The output of stable isotope enrichment results calculated using Polly LC-MS/MS labeled workflow Phi (Beta).

In addition, the workflow also calculates the fractional enrichment of metabolite species and performs a background correction. The embedded search function allows the table to be filtered to only display the desired metabolite. The data can be represented graphically using the built-in visualization tools, Figure 4 shows the natural abundance corrected data for lactate. The natural abundance corrected data (represented as peak area, Figure 4A) for lactate and its isotopologues show that all isotopologue species have increased in activated T-cells (blue) compared to rested T-cells (orange). The fractional enrichment data (represented as the percentage of total lactate pool) show similar amounts of M+3 species between activated and resting T-cells (Figure 4B). An increased relative flux from glucose through lactate is suggested in activated T-cells by the lower dilutions from other non-labeled pathways. Further examination of the fractional enrichment profile of TCA intermediates show complex labeling patterns (Figure 4C and D). Citrate has an increased amount of ^{13}C incorporated into the M+2 species in general but there is more dilution between citrate and malate in activated T-cells indicating. While these data are suggestive of different utilization patterns of citrate, a more definitive interpretation requires the interpretation of positional enrichment differences between precursor and product metabolites (see below). All the data (tables and graphs) can be easily exported in a .csv or .png picture file, respectively. This fully automated workflow removes

the time consuming rigorous manual labor involved in processing these types of experiments.

Phi – Flux calculations

Once the data is corrected for naturally occurring isotopes, the individual flux rates (Phi or ϕ) can be calculated. This is accomplished in the Polly Phi (Beta) tab that contains three subtabs: Calculate Phi, Results: Glucose Labeled, and Results: Generic Labeled. The Calculate Phi tab is used to define the labeled isotopes used and to display the different cohorts. Also, Polly offers customizable options for metabolites with different labeling schemes and fragmentation patterns. The two results tabs display the phi values in table and graph form for glucose and generic (used for other labeled precursors like glutamine), respectively.

In this fluxomics experiment, only uniformly ^{13}C labeled glucose ($[\text{U-}^{13}\text{C}_6]\text{-D-glucose}$) was used. Figure 5 depicts the results for the glucose labeled output with the different phi values (rows) for each sample (columns) listed. Here 45 individual phi values and positional isotopomers were calculated when using $[\text{U-}^{13}\text{C}_6]\text{-D-glucose}$ as the tracer and these can be expanded to include others by user. Polly has built-in visualization tools to graphically display the flux data. Representative graphs with the expression formulas are shown to highlight the changes in metabolic flux between activated and rested cells (Figure 6). Activated T-cells

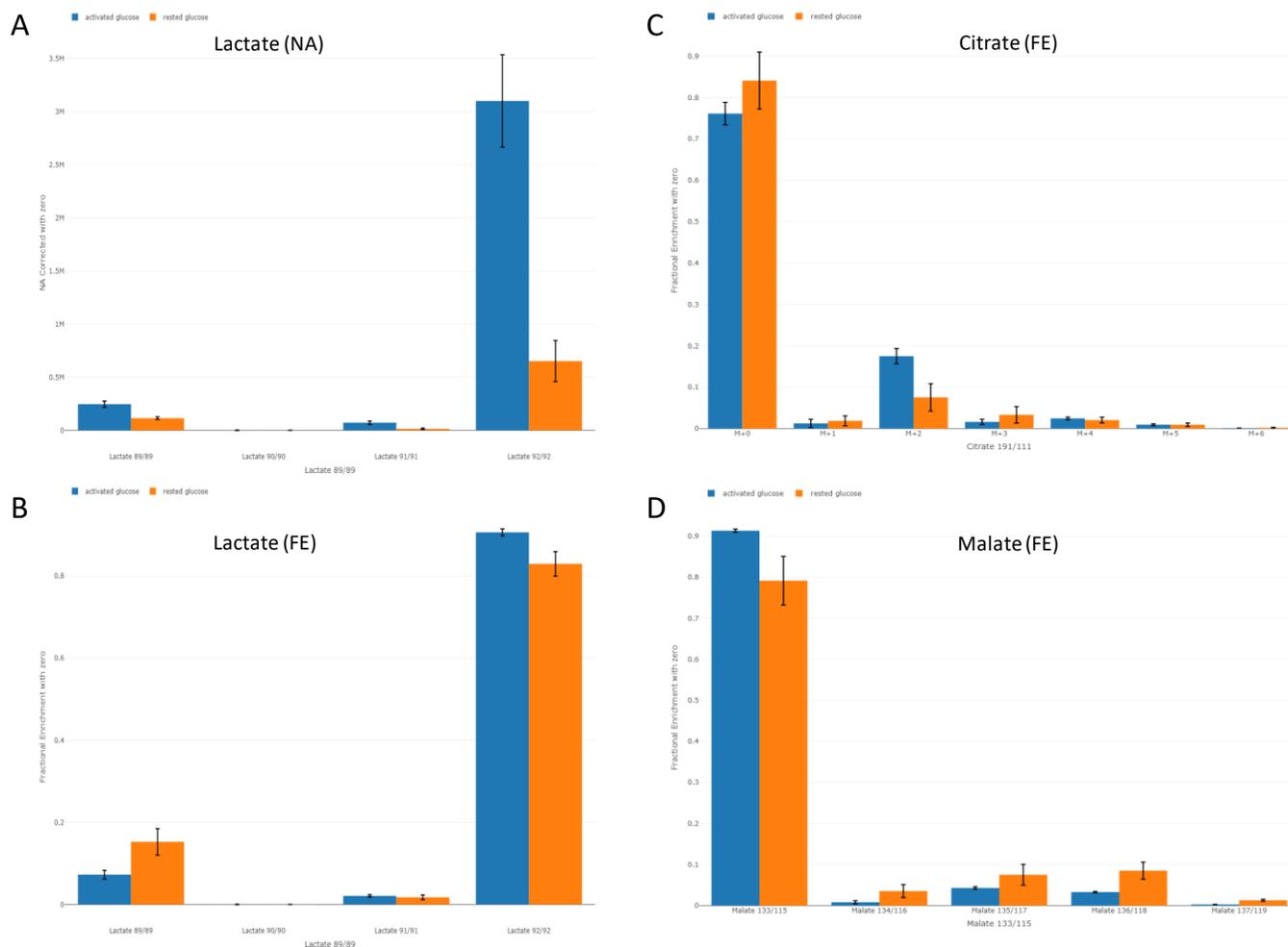


Figure 4. Visualization of natural abundance correction (NA) and fractional enrichment (FE). Representative graphs using Polly's built-in visualization tools for natural abundance correction (NA) and fractional enrichment (FE) in activated (blue bars) and rested (orange bars) T-cells. (A) depicts the natural abundance corrected isotope profile for lactate. The data is reported as average peak area. (B, C, and D) are the fractional enrichment plots for the isotope profiles of lactate, citrate, and malate, respectively. The data is reported as % of total metabolite pool. Each cohort had an n value = 6.

were observed to have a 2.5-fold increased enrichment in the pyruvate dehydrogenase (PDH) derived carbons of citrate (Figure 6A) despite only a slightly greater enrichment in lactate (Figure 4B). This identifies a greater contribution of carbohydrate oxidation to overall TCA flux in the activated cells. In contrast, there is a 70% greater loss of ^{13}C enrichment between Acetyl CoA (carbons C1,2) and those same carbons in glutamate (C4,5, Figure 6C). The same observations made by an independent calculation quantitatively validated this dramatic finding (Figure 6B). To represent the data in a biological context, metabolic flux changes that occurred in response to T-cell activation were mapped to central carbon pathways (Figure 1). Representing the data in this fashion can be useful for understanding metabolic changes, nutrient utilization, and pathway directionality.

Discussion

T-cells undergo a metabolic reprogramming when activated by a stimulus event. Using $[\text{U-}^{13}\text{C}_6]\text{-D-glucose}$, these metabolic changes were assessed after 24 hours of activation. An elevated flux of glucose through glycolysis (compared to other pyruvate generating fluxes) was observed in activated T-cells with the accumulation of ^{13}C -carbon in lactate. The dependence on glycolysis, which can rapidly generate energy, is thought to be in response to the cell's accelerated growth in mounting an immune response. Glucose was also utilized to generating citrate via PDH by a greater extent, but reduced label incorporations were seen in other TCA cycle intermediates and metabolites (malate and glutamate). One explanation of this observation is that a dilution of ^{13}C -carbon is occurring after citrate. Consistent with this, it has been reported that activated T-cells exhibit increased uptake of exogenous glutamine. Unlabeled glutamine could be

responsible for the observed dilution entering the TCA via glutamate to α -ketoglutarate exchange. A flux experiment using labeled glutamine (for instance, [1,2- ^{13}C]-L-glutamine) would be needed to provide an orthogonal confirmation.

This experiment was used to showcase the utility of a targeted fluxomics approach to better understand the dynamic metabolic changes that occur during T-cell activation. The sensitivity and speed of the QTRAP 6500+ System enabled the highest quality data to be collected for the 100+ MRMs within the method. The orthogonal separation of the SelexION Technology was instrumental in separating many of the isobaric compounds in glycolysis and the TCA cycle.

The Polly LC-MS/MS labeled workflow removed many of the barriers and bottlenecks associated with processing fluxomic data. This was accomplished by streamlining the processing from peak integration to natural abundance correction. In addition to reducing data processing time, the automated workflow eliminated user derived errors commonly associated with multi-tab spreadsheets. Another positive feature of Polly is the visualization tools. Comparisons between different cohorts can quickly be selected and graphed. The data and graphs are easily exported for use in manuscripts, posters, or presentations. The reducing the time from sample to graph allows the user to spend more time in understanding the biological impact of their experiment.

Phi Results for Glucose Labeled (for TCA Cycle)

Identifier	20170320_TcellTimeCourse-4uL-Activated Glucose labeledovernight-1 (sample 36)	20170320_TcellTimeCourse-4uL-Activated Glucose labeledovernight-2 (sample 49)	20170320_TcellTimeCourse-4uL-Activated Glucose labeledovernight-3 (sample 96)	20170320_TcellTimeCourse-4uL-Activated Glucose labeledovernight-4 (sample 141)	20170320_TcellTimeCourse-4uL-Activated Glucose labeledovernight-5 (sample 14)	20170320_TcellTimeCourse-4uL-Activated Glucose labeledovernight-6 (sample 120)	20170320_TcellTimeCourse-4uL-Rested Glucose labeledovernight-1 (sample 5)	2017 lab
1 OAA_PC	0.035206929854656	0.0242652790623897	0.00894827693580341	0.0274428700470382	0.00744855430687618	0.0305472809392229	0.00985746159824193	
2 OAA_TCA	0.013487075303273	0.0140736494490532	0.0234111296173876	0.0189613182042456	0.0347298431582876	0.0274932172536441	0.0756029971733011	
3 Phi_Cit_Glut	0.16916637600279	0.376320422535211	1.18089271730619	0.0684993240198287	0.345960748516659	2.75585585585586	1.50709219858156	
4 Cit_PC	0.0118441674	0.00574544585	0.0057700321689109	0.0156576880005633	0.0018121376	0.0167009831839249	0.0010431846	
5 Phi_AccitG	0.127148106072331	0.190363211335782	0.1444447914695073	0.136892814991859	0.129900677170141	0.148530092616337	0.395058191507689	
6 Phi_CRG1	0.471942759445791	0.571822360250276	0.404815298192145	0.419190654458122	0.54420464159112	0.454566041671536	2.34720378910596	
7 OAA_M2	0.104180890591419	0.114045348778846	0.123747874908227	0.107705619592119	0.120064866515781	0.109059267677776	0.19919367956165	
8 OAA_M3	0.0486940051579291	0.038338928511443	0.032359406553191	0.0464041882512838	0.0421783974651637	0.0580404981928669	0.0854604587715431	
9 OAA_M4	0.00524380843828657	0.00726655980767701	0.00774519065418644	0.00542993075722956	0.00394759843641227	0.00609040981533117	0.0304487801181258	
10 Phi_MO1	2.36237847146076	2.65221741346153	2.56206780348296	2.54623214165765	2.9866882217856	2.74708482815557	2.75509930237414	

Figure 5. Phi results for TCA cycle calculated from MIMOSA deconvolved isotopomers from [U- $^{13}\text{C}_6$]-D-glucose tracer labeling of T-Cells in Polly Phi (beta). Shown are 10 of the 45 Phi calculations (identifier column) for the different T-Cell samples. Individual mathematical descriptions of the different Phi shown in a different tab (see Figure 6). Data from individual samples (identifier descriptions provided) are shown as fractional values (multiply x 100 for percent).

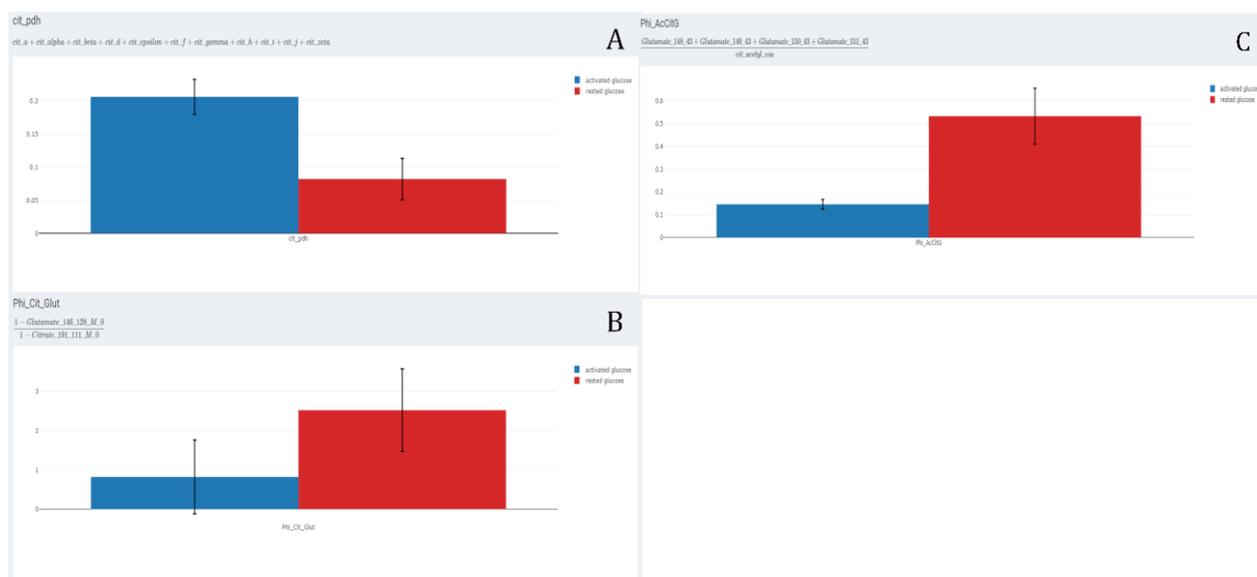


Figure 6. Representative graphs using Polly's built-in visualization tools for calculated fluxes (Phi values) for activated (blue) and rested (red) T-cells. (A) A greater enrichment in the pyruvate dehydrogenase (PDH) derived carbons of citrate was observed in activated T-cells. The next segment of the TCA cycle showed the opposite, namely there was a greater entry of glutamate carbon (via GDH or transamination pathways) in activated T-Cells as demonstrated by two independent calculations from citrate to glutamate (**B**) Φ_{Cit_Glut} and from Acetyl-CoA to citrate to glutamate (**C**) Φ_{AcCitG} , respectively. Each cohort had an n = 6.

References

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