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QUANTIFYING METABOLIC TRANSFER MEDIATED BY EXTRACELLULAR VESICLES USING EXO-MFA: AN INTEGRATED EMPIRICAL AND COMPUTATIONAL PLATFORM

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Abstract

Extracellular vesicles (EVs) are ubiquitous nanoscale particles released from many different types of cells. They have been shown to contain proteins, DNA, RNA, miRNA, and most recently, metabolites. These particles can travel through the intercellular space and bloodstream to have regulatory effects on distant recipients. When an EV reaches a target cell, it is taken up and degraded to release its contents for utilization within the cell. In addition to regulatory effects, EVs have been shown to supplement the high metabolic demands of recipient cells in a nutrient-deprived tumor microenvironment. We developed an integrated empirical and computational platform to quantify metabolic contribution of source cell-derived EVs to recipient cells. The versatile Exo-MFA software tool utilizes ¹³C stable-isotope tracing data to quantify the metabolic contributions of EVs from a source cell type on a recipient cell type. This is accomplished by creating EV-depleted culture medium, producing isotope-labeled EVs from the source cells, isolating the labeled EVs from the culture supernatant, culturing the recipient cells in the presence of the labeled EVs, and measuring the resulting metabolite levels across several time points.

Keywords

Extracellular vesicles; exosomes; Exo-MFA; stable-isotope tracing; 13-carbon metabolic flux analysis; multicellular metabolic flux analysis

1 Introduction

Among the wide range of intercellular interaction mechanisms, microvesicles secreted by cells are an important apparatus of communication between cells. **Extracellular vesicles (EVs)** have been documented as part of fundamental biological machinery as well as being involved in disease progression of aggressive cancers [1-4]. In this chapter, we focus on EVs – also referred to here as exosomes – which are 30-150 nm in diameter and transport proteins and nucleic acids including miRNA intercellularly including distant targets via the

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⁴Notes

bloodstream [5, 6]. Recently, using ^{13}C tracer experiments our lab provided compelling evidence that these EVs also transport free metabolites that are directly incorporated into recipient cells' central carbon metabolite pools [4]. However, a quantified insight into the contribution of metabolite cargo towards rescuing recipient cells from nutrient deprivation was lacking at the time. To address this, we designed a novel paradigm, **Exosome-mediated metabolic flux analysis (Exo-MFA)**, to predict fluxes involved in metabolite trafficking from source cells to recipient cells [7]. The protocols described here can be applied to any type of cells that exchange biological cargo via exosomes or any other type of EV.

Exo-MFA integrates a novel experimental protocol using ^{13}C -labeled substrates with an enhanced metabolic flux analysis to provide insight into metabolic crosstalk. Exo-MFA utilizes the fundamentals of the **13-carbon metabolic flux analysis (^{13}C -MFA)** algorithm and tracer experiments [8, 9], traditionally used for single cell systems that only exchange metabolites with their medium, to provide an enhanced platform to analyze metabolite fluxes in multicellular systems. Exo-MFA is designed to quantify intracellular fluxes and flux of metabolites via extracellular vesicles (EVs) from '*source cells*' to '*recipient cells*' (Figure 1). The paradigm described herein considers two important processes that occur in the system; (i) packaging of metabolite cargo into EVs and secretion of EVs within *source cells* and (ii) internalization of EVs and release of cargo within *recipient cells*. Source cells are cultured in medium containing stable ^{13}C isotope-labeled tracer substrates in order to obtain EVs with ^{13}C -labeled cargo. The ^{13}C -labeled EVs are then introduced into cell medium, which release their labeled cargo into recipient cells. Exo-MFA describes a ^{13}C -MFA problem that includes packaging fluxes describing the generation of EVs and utilizes data from (i) tracer experiments within source cells and EVs, (ii) extracellular fluxes of source cells and (iii) growth rate measurements. Source cells are cultured in medium with the labeled tracers for 72 hours to allow for sufficient production of EVs and enrichment of glycolytic and TCA cycle intermediates along with de novo synthesized amino acids. EVs enriched with labeled metabolites are isolated from the spent medium and their cargo is analyzed using GC-MS. Exo-MFA then considers the transient nature of EV internalization and utilizes data from time-series isotope-labeled EV experiments, measurements of extracellular fluxes, and composition of EV metabolite cargo to estimate intracellular fluxes in recipient cells that internalize EVs from the medium. Exo-MFA for recipient cells is set up as a time-series ^{13}C -MFA problem and solved for measurements sampled at various times such as 3, 6, 12 and 24 hours (Figure 1).

Modeling packaging fluxes in source cells

In order to obtain sufficient information for estimating metabolite packaging fluxes into EV cargo, the rate of EV secretion along with extracellular fluxes are empirically determined. If each source cell is assumed to produce EVs at a constant rate of r (mg EV/mg protein/h), the total EVs secreted at the end of a tracer experiment is described by Equation (1), with the knowledge that exponential growth rate of source cells is μ (hr^{-1}) and seeding density of source cells is X_0 (cells):

$$\frac{d(\text{Exo})}{dt} = rX = rX_0e^{\mu t} \dots \quad (1)$$

Integrating Equation (1) will give the expression for total number of EVs produced as given by Equation (2)

$$Total\ exosomes\ [mg\ exosome] = \int d(Exo) = \int rX_0 e^{\mu t} dt = \frac{rX_0 e^{\mu t}}{\mu} + C \dots \quad (2)$$

Assuming that there are no EVs in fresh medium at the beginning of the experiment, the constant of integration can be evaluated at $t=0$ and therefore, r can be estimated from Equation (2) once total amount of EVs produced at the end of the experiment is measured. The abundance of each metabolite in EV cargo can be estimated by analyzing EVs in the GC-MS and Equation (3).

$$\begin{aligned} Total\ i^{th} exosomal\ metabolite\ [\mu mol] \\ = (P_i \left[\frac{\mu mol}{mg\ exosome} \right]) \cdot (Total\ exosome\ [mg\ exosome]) \dots \end{aligned} \quad (3)$$

However, measurements for all metabolites that are packaged in cargo are not practical to obtain empirically. For this purpose, Exo-MFA has the ability to predict the packaging fluxes for set of metabolites not measured via targeted GC-MS analysis. This is achieved by introducing P_i as unknown parameter for metabolites that are postulated to contribute to the cargo but not measured *a priori*. Furthermore, even for metabolites that are measured, contribution of the same metabolite from cytosolic and mitochondrial compartments to EV cargo cannot be inferred from Equation (3). Exo-MFA also includes isotopomer balances in EVs in addition to intracellular isotopomer balances described in the ^{13}C -MFA formulation (described in detail in [7]).

Mass balance for intracellular metabolites that are packaged into EVs within source cells is described under the steady state assumption by Equations (4-5), where C_i is the intracellular concentration of metabolite i ; S_i is the stoichiometric vector corresponding to metabolite i and the parameter introduced to represent EV packaging flux is v_i^{exo} . For metabolites that exist in multiple compartments, mass balances in Equations (5) are defined for each subcompartment the same way as they are for metabolites in single compartments (Equations (4)), however the balance for EV packaging flux is modified as in Equation (5c) to include contribution from both compartments.

$$\frac{dC_i}{dt} = S_i \vec{v} - v_i^{exo} = 0, i \in M_{1C} \dots \quad (4a)$$

$$v_i^{exo} - rP_i = 0, i \in M_{1C} \dots \quad (4b)$$

$$\frac{dC_{i,cyt}}{dt} = S_{i,cyt} \vec{v} - v_{i,cyt}^{exo}, i \in M_{2C} \dots \quad (5a)$$

$$\frac{dC_{i,mit}}{dt} = S_{i,mit} \vec{v} - v_{i,mit}^{exo}, i \in M_{2C} \dots \quad (5b)$$

$$v_{i,cyt}^{exo} + v_{i,mit}^{exo} - rP_i = 0, i \in M_{2C} \dots \quad (5c)$$

Further, the isotopomer balance for metabolites which are measured in source cells and EVs via GC-MS is described in a generalized form in Equation (6). The isotopomer distribution of metabolite i in the EV is a combination of isotopomer distributions derived from multiple compartments that is proportional to the packaging fluxes originating from each compartment.

$$v_{i,cyt}^{exo} \cdot \begin{pmatrix} y_0 \\ \vdots \\ y_{2^n-1} \end{pmatrix}_{i,cyt} + v_{i,mit}^{exo} \cdot \begin{pmatrix} y_0 \\ \vdots \\ y_{2^n-1} \end{pmatrix}_{i,mit} - rP_i \cdot \begin{pmatrix} y_0 \\ \vdots \\ y_{2^n-1} \end{pmatrix}_{i,exo} = 0 \dots \quad (6)$$

Here, M_{1C} and M_{2C} are set of metabolites that exist in single compartment or two compartments, respectively; $v_{i,cyt}^{exo}$, $v_{i,mit}^{exo}$ refer to packaging fluxes of i^{th} metabolite from the single compartment, cytosolic compartment or mitochondrial compartment, respectively; $\vec{y}_{i,cyt}$, $\vec{y}_{i,mit}$, $\vec{y}_{i,exo}$ are isotopomer distribution vectors (IDVs) of i^{th} metabolite existing in the cytosol, mitochondria or EV. (Equations 4-6) are included in the Exo-MFA algorithm along with 13C-MFA mass balance and isotopomer balance constraints. The objective function for Exo-MFA is modified from the 13C-MFA objective function to consider the error residuals of additional measurements in source cells and EVs, i.e., EV secretion rate, metabolite levels in EVs and **mass isotopologue distributions (MID)** in EVs.

Modeling cargo release in recipient cells

Exo-MFA modifies the metabolic model for recipient cells to include EV internalization and release of metabolite cargo that contributes to the endogenous metabolite pools. The rate of internalization is considered to be $u(t)$ (mg EV/mg protein/h). Internalization is assumed to be time-dependent due to dependence on extracellular concentration of EVs and the transient nature of the nutrient-deprived recipient cells. The content of EVs, however, is assumed to be consistent throughout the process of packaging, transport and internalization. Release of cargo into recipient cells is slightly more complex than packaging, since (i) not all metabolites are utilized in the same way, and (ii) intracellular metabolic fluxes are not at steady-state. For this purpose, the cargo is categorized according to their utilization, (i) central carbon metabolites that are incorporated directly into central carbon metabolism, (ii) essential amino acids that are incorporated only into biomass. The mass balance for central carbon metabolites are formulated in Equations (7). Cargo release fluxes are proportional to rate of internalization $u(t)$ and the intra-EV composition P_i , therefore the flux term becomes $u(t)P_i$ (μmol/mg protein/h). Equation (7) describes the mass balance of metabolite i derived from EV cargo and S_i is the stoichiometric vector corresponding to that metabolite describing the intracellular reactions it is involved in. Equation (8) represent the isotopomer balance equations as described by the Isotopomer Mapping Matrix algorithm [10, 11], but

with a small modification that includes the term $u(t)P_i y_{i,exo}$ that represents the influx of isotopomers of metabolites derived from EV cargo.

$$\frac{dC_i}{dt} = u(t)P_i + S_i \vec{v} = 0, \quad i \in M_{CCM} \dots \quad (7)$$

$$\begin{aligned} \frac{d}{dt} \left(C_i \begin{pmatrix} y_0 \\ \vdots \\ y_{2^n-1} \end{pmatrix}_{i, cell} \right) &= u(t)P_i \begin{pmatrix} y_0 \\ \vdots \\ y_{2^n-1} \end{pmatrix}_{i, exo} + \sum_{j=1}^N S_{ij} \\ &\cdot v_j \left(\prod_{\substack{S_{ij} > 0 \\ k, S_{kj} < 0}} IMM_{k \rightarrow i} \cdot \begin{pmatrix} y_0 \\ \vdots \\ y_{2^n-1} \end{pmatrix}_{k, cell} \right) \\ &+ \sum_{j=1, S_{ij} < 0}^N S_{ij} \cdot \begin{pmatrix} y_0 \\ \vdots \\ y_{2^n-1} \end{pmatrix}_{i, cell} = 0, \end{aligned} \quad (8)$$

C_i is the total intracellular concentration of metabolite i . The objective function of Exo-MFA in recipient cells, is modified to include residuals of metabolite levels in EVs, and MID in both recipient cells and EVs.

2 Materials

1. Fetal bovine serum (FBS)
2. Ultracentrifuge
3. Vacuum Centrifuge
4. Gas Chromatography-Mass Spectrometer (GC-MS)
5. HP-5MS or equivalent column
6. Nanoparticle Tracking Analysis (NTA) Instrument
7. 0.2 micron filter
8. Culture medium
9. Isotope labeled substrates
10. Phosphate Buffered Saline (PBS)
11. RIPA Buffer
12. Bicinchoninic (BCA) Kit
13. Wako Glucose Kit

14. HPLC-Grade Substrate Standards
15. Methanol
16. Norvaline
17. Chloroform
18. 0.9% saline in water
19. 2% methoxyamine hydrochloride in pyridine
20. MBTSTFA+1% TBDMCS
21. Desktop computer or access to computational cluster with MATLAB license
22. MATLAB 2016a or later, with licenses for Optimization and Parallel Computing Toolboxes
23. Artelys Knitro 10 or later (or MATLAB's in-built Optimization Toolbox and Parallel Computing Toolbox)
24. Microsoft Office Suite (or any spreadsheet software that can save files in the .XLS or .XLSX formats)

3 Methods

3.1 Fetal Bovine Serum Depletion

1. Load fetal bovine serum into clean, sterile ultracentrifuge tubes. Tubes should be filled completely and balanced precisely. Centrifuge at 100,000-120,000xg for 19 hours at 4°C (*see* Note 1) (Figure 2A).
2. Carefully remove the light-colored upper layers into fresh tubes, discarding the lower dark layer and pellet. The upper layer consists of approximately 90% of the serum.
3. Sterile filter the depleted serum.
4. Use immediately or aliquot and store at -20 °C (*see* Notes 2 and 3).

3.2 Preliminary Characterization of Extracellular Vesicles and Cell Lines

1. Seed donor cells and grow to 70% confluency in recommended culture medium (*see* Note 4).
2. Aspirate culture medium and wash cells twice with PBS

¹The precise centrifugation speed and time required to effectively precipitate a particle cannot be determined solely from the relative centrifugal force (RCF). Instead it must be determined by the geometry of the rotor as well as the speed of rotation expressed as the k-factor. The optimal speed for a given rotor should therefore be determined uniquely for each application. RCF quoted here is offered as a general guideline.

²Depletion efficiency can be verified via Nanoparticle Tracking Analysis.

³Depleted FBS can also be purchased.

⁴EV production rates can vary dramatically by cell type and origin. Before beginning a new experiment, the donor cells should be characterized to select the optimal cell number. As a general guideline, high-production cells like cancer can be grown in as few as 5 T-75 flasks while low-production cells like fibroblasts may need as many as 15 T-160 flasks.

3. Add culture medium containing EV-depleted FBS
4. After 48 hours, remove the spent medium from the flasks into centrifuge tubes for EV isolation (*see* Note 5).
5. Quantify donor cells via protein assay after 48 hours for normalization

3.3 Isolation of Extracellular Vesicles from Conditioned Medium via Ultracentrifugation

1. Centrifuge spent culture medium containing EVs from step 4 of the previous section at 300xg for 10 minutes at 4 °C to remove cell debris.
2. Transfer the supernatant to fresh centrifuge tubes, careful to avoid disturbing the pellet. Centrifuge the supernatant at 10,000xg for 30 minutes at 4°C to remove larger vesicles.
3. Transfer the supernatant into clean, sterile ultracentrifuge tubes, careful to avoid disturbing the pellet. Tubes should be balanced precisely. If tubes are not full to maximum volume, dilute with PBS. Centrifuge at 100,000-120,000xg for 90 minutes at 4 °C (*see* Notes 1 and 6) (Figure 2B).
4. Aspirate the supernatant, careful to avoid disturbing the pellet. Resuspend the pellet in a full volume of PBS at 4 °C and repeat the centrifugation described in step 3 with the tube in the same orientation. Aspirate the supernatant again and resuspend the pellet of EVs in an appropriate volume of solution for the desired application (*see* Note 7).
5. For protein quantification, the pellet can be resuspended directly in 100 µL RIPA buffer and quantified via BCA assay following manufacturer instructions (*see* Note 8).
6. For particle number, the pellet can be resuspended in 100 µL of PBS and diluted for counting via Nanoparticle Tracking Analysis (*see* Note 9).
7. For treatment of cells, the pellet can be resuspended in 100 µL of culture medium and immediately applied to the recipient cells. After 24 h of treatment the recipient cells should be quantified via protein assay for normalization.

3.4 Isotopic Labeling of Donor Cells for Extracellular Vesicle Production

1. Seed donor cells and grow to 70% confluency in recommended culture medium (*see* Note 4).

⁵. At least 48 h are necessary for EVs to accumulate in the medium. After longer than 48 h the EVs can be taken up again and degraded by the source cells, preventing increased yield.

⁶. The EV pellet can be difficult to observe. It is helpful to mark the centrifuge tube where the pellet is expected to assist in maintaining the orientation and avoiding disturbances.

⁷. EVs should be characterized according to the standards defined in the Minimal Information for Studies of Extracellular Vesicles [12]. This includes a determination of particle number and protein content as described here in addition to characterization of typical protein markers.

⁸. The micro BCA kit is specifically designed for low sample concentrations (0.5 – 20 µg/mL) and is particularly well-suited for measuring low EV protein concentrations.

⁹. For an uncharacterized EV source, several dilutions must be tested to determine the optimal concentration for measurement. Particle number can be estimated from protein concentration using the ratio of 4.9 µg protein/10⁹ particles but should be verified independently.

2. Aspirate culture medium and wash cells twice with PBS
3. Add culture medium with extracellular vesicle depleted FBS and isotope-labeled substrates of interest replacing the unlabeled at the original concentrations. Reserve 1 mL of medium for metabolic analysis and store at -80°C (Figure 3A).
4. After 48 hours, remove the spent medium from the flasks into centrifuge tubes for EV isolation (*see Note 5*). Reserve 1 mL of medium for metabolic analysis and store at -80°C .
5. Extract intracellular metabolites immediately. Wash with cold saline once and then quench the cells in cold methanol (*see Note 10*). Add an equal volume of water with 1 μg of norvaline and scrape cells thoroughly.
6. Pipet mixture into fresh tubes and add two volumes of chloroform. Vortex at 4°C for 30 min and centrifuge at 5,000xg for 10 min at 4°C . Remove the upper aqueous layer containing polar metabolites into a new tube. Dry via vacuum centrifugation. Store dried samples at -80°C .

3.5 Isolation of 13-Carbon Labeled Extracellular Vesicles from Conditioned Medium via Ultracentrifugation

1. Centrifuge spent culture medium containing labeled EVs from step 4 of the previous section at 300xg for 10 min at 4°C to remove cell debris.
2. Transfer the supernatant to fresh centrifuge tubes, careful to avoid disturbing the pellet. Centrifuge the supernatant at 10,000xg for 30 min at 4°C to remove larger vesicles.
3. Transfer the supernatant into clean, sterile ultracentrifuge tubes, careful to avoid disturbing the pellet. A separate tube containing 300 μg protein based on earlier quantification should be aliquoted for direct analysis of EV samples. Tubes should be balanced precisely. If tubes are not full to maximum volume, dilute with PBS. Centrifuge at 100,000-120,000xg for 90 minutes at 4°C (*see Notes 1 and 6*) (Figure 2B).
4. Aspirate the supernatant, careful to avoid disturbing the pellet. Resuspend the pellet in a full volume of PBS at 4°C and repeat the centrifugation described in step 3 with the tube in the same orientation.
5. For treatment of recipient cells, the pellet can be resuspended in culture medium with EV depleted FBS. The EVs should be diluted to approximately 200 μg protein/mL or 40×10^9 particles/mL for treatment based on earlier quantification. Reserve three replicate samples of EVs equivalent to 100 μg protein each resuspended in 150 μL water containing 1 μg norvaline for metabolic analysis, stored at -80°C .

¹⁰.If PBS is used to wash in place of saline, the phosphate can be detected on the GC-MS and obscure the measurements.

3.6 Treating Recipient Cells with Isotopically Labeled Extracellular Vesicles

1. Seed recipient cells at desired confluency in a 6-well plate and leave overnight. Then replace culture medium with fresh medium spiked with ^{13}C labeled EVs (Figure 3B).
2. At 3, 6, 12, and 24 hours extract intracellular metabolites. Reserve 1 mL from each well and store at $-80\text{ }^{\circ}\text{C}$ for metabolic analysis. Aspirate remaining medium. Wash with cold saline once and then quench the cells in cold methanol. Add an equal volume of water with 1 μg of norvaline and scrape cells thoroughly.
3. Pipet mixture into Eppendorf tubes and add two volumes of chloroform. Vortex at $4\text{ }^{\circ}\text{C}$ for 30 min and centrifuge at 5,000xg for 10 min at $4\text{ }^{\circ}\text{C}$. Remove the upper aqueous layer containing polar metabolites into a new tube. Dry via vacuum centrifugation. Store dried samples at $-80\text{ }^{\circ}\text{C}$.

3.7 Extracellular Vesicle Sample Preparation

1. Transfer 75 μL of cold methanol to each EV sample after thawing from $-80\text{ }^{\circ}\text{C}$. Reserve 20 μL of the resulting solution for protein assay.
2. Add 150 μL of cold chloroform to each sample and vortex for 30 min at $4\text{ }^{\circ}\text{C}$. Centrifuge at 5,000xg for 10 min at $4\text{ }^{\circ}\text{C}$ to separate the phases. Remove the upper aqueous layer containing polar metabolites into a new tube. Dry via vacuum centrifugation. Store dried samples at $-80\text{ }^{\circ}\text{C}$.

3.8 Media Sample Preparation

1. Transfer 200 μL of medium collected from fresh ^{13}C tracer medium, donor cell conditioned medium, and recipient cell conditioned medium after thawing from $-80\text{ }^{\circ}\text{C}$.
2. Add 10 μL of water containing 1 μg norvaline in water to each tube as internal standard
3. Add 800 μL of pre-chilled methanol to each tube and vortex for 10 min
4. Allow samples to deproteinize for 2 h at $-20\text{ }^{\circ}\text{C}$
5. Centrifuge at 14,000xg for 10 min at $4\text{ }^{\circ}\text{C}$ to collect protein at the bottom of the tube and transfer the supernatant to fresh tubes. Dry the samples via vacuum centrifugation and store the dried metabolites at $-80\text{ }^{\circ}\text{C}$.
6. From remaining medium, quantify glucose via Wako Glucose Kit following manufacturer instructions.

3.9 GC-MS Analysis of Metabolites

1. Prepare external standard curve mixture including relevant amino acids, glycolytic intermediates, and TCA cycle metabolites via serial dilution (*see Note 11*).

2. Retrieve polar metabolite samples from storage at -80°C including donor cells, EVs, recipient cells, and media samples. Dry samples and external standards via vacuum centrifugation briefly to remove condensation (*see* Note 12).
3. Derivatize samples by dissolving in 30 μL of 2% methoxyamine hydrochloride in pyridine, sonicating for 10 min, and incubating at 37°C for 2 h.
4. Add 45 μL of MBTSTFA+1% TBDMCS and incubate at 55°C for 1 h.
5. Transfer into vials containing glass inserts for GC-MS measurement.
6. The GC-MS should be equipped with a HP-5MS column or equivalent. The method parameters are as follows: helium carrier gas with flow of 1 mL/min. Injection volume of 1-2 μL at 270°C . Oven temperature of 100°C for 3 min raised at $5^{\circ}\text{C}/\text{min}$ to 300°C over 40 min and then held for 5 min. Solvent delay of 6-10 min depending on when the initial saturating signal is observed to have dropped. MS source is set to 230°C , and MS quadrupole is set to 150°C . MS detector operated in scan mode from 50-450 m/z.

3.10 Estimating extracellular fluxes

1. Estimate the growth rate of source cells and recipient cells from assays performed in steps 3.2.5 and 3.3.7. Estimate growth rate, μ , assuming exponential growth by fitting to Equation 9.

$$X = X_0 e^{\mu t} \dots \quad (9)$$

2. For *source cells*, using the measurement of extracellular metabolite concentrations from step 3.4.3 at the start time (fresh medium) and step 3.4.4 at time T (spent medium), estimate the extracellular flux v_{ext} from the integrated form of Equation 10.

$$\int_{C_0}^{C_T} dC = -v_{ext} X_0 \int_0^T e^{\mu t} dt \dots \quad (10)$$

3. Repeat step 3.10.3 for all extracellular metabolites that you will consider in the Exo-MFA model
4. For *recipient cells*, extracellular fluxes are measured soon after treatment with culture medium with EVs. Thus, their metabolism is not at steady state and their extracellular fluxes will not be constant over the course of the experiment. Their extracellular fluxes extracellular metabolites are derived analytically and integrated for a set of unknown parameters as in Equation 11 (*see* Note 13).

¹¹The standard curve may need to be adapted based on the experimental conditions. A typical curve would span 0.1-10 nmol of each metabolite.

¹²Any amount of water will prevent derivatization. It is imperative to ensure samples are completely dry before continuing with analysis.

¹³For each extracellular metabolite $v_{ext}(t)$ was assumed to be a linear function of time. For our experimental design, the parameters were estimated by fitting Equation 11a to extracellular concentrations measured at 0, 3, 6, 12 and 24 hours of culture for recipient

$$v_i(t) = a + bt \dots \quad (11a)$$

$$\int \frac{dC_i^{ext}}{dt} dt = - \int v_i(t) X_0 e^{\mu t} dt \dots \quad (11b)$$

3.11 Using Exo-MFA to quantify intracellular and EV-mediated metabolic flux

1. Build and curate a model defining the metabolic network in donor and recipient cells. A template for the model input files can be downloaded from supplemental files.
2. The first column is ignored by the Exo-MFA program, and is meant to help users identify reactions. The second column defines the reaction behavior: I, irreversible; R, reversible; E, exchange or boundary. The third column semantically defines the stoichiometry of each reaction. The fourth column defines the carbon atom transitions. Metabolites and compartments are defined by the user but must follow the format: Metabolite_Compartment (*see* Note 14).
3. The empirically measured data for *source cells* is compiled in an input Excel file, for which the template can be downloaded from supplemental files.
4. For *source cells*, enter the stable-isotope tracers used and their carbon labeling pattern in the sheet 'tracer' (*see* Note 15). Enter measured extracellular fluxes in the first column of the sheet 'flux', followed by the standard deviation and name of the metabolite (as referred to in the model file). Enter the mass isotopologue distribution data for intracellular metabolites and intra-EV metabolites in the first column of the sheet 'MID', followed by the standard deviation of each measurement, name of the metabolite and the carbon chain. These are the measurements obtained in Section 3.9. In the sheet 'exo', list the metabolite names (without denoting the compartment) that are detected in the EV cargo in the first column, followed by the compartments in the source cell which are possible sources of those metabolites (*see* Note 16). The third and fourth columns are the measured abundance and standard deviations of the metabolites in the EV cargo. In the 'exo_rate' sheet, report the rate of secretion of EVs by

cells. The $v_{ext}(t)$ function can be a polynomial as well if the fit for a linear model is not good. However, additional parameters will need to be fitted for higher order polynomials, and users should take care that the number of measurements must be larger than the number of parameters being fitted.

¹⁴We consider three compartments in the source cells: c, cytosol; m, mitochondria; x, extracellular; and d, a dilution source of unlabeled metabolites that contribute to EV cargo. The user can add more compartments or merge cytosolic and mitochondrial compartments, depending on the complexity of the model. The metabolites from compartment 'd' only affect the 13-carbon enrichment of EV cargo and do not interfere in the estimation of packaging flux metabolites from source cells' cytosolic and mitochondrial compartments to EV cargo. This is included to account for the production of EVs when intracellular metabolites are not saturated with 13-carbon from the stable-isotope tracers.

¹⁵Tracer labeling pattern follows a binary pattern, for e.g., U-¹³C₆-Glucose containing six 13-carbon atoms is represented as "111111" and 5-¹³C₁-Glutamine is represented as "00001".

¹⁶Zhao et al. and Achreja et al. have previously shown the presence of amino acids, glycolytic intermediates and TCA cycle intermediates which exist in multiple compartments in source cells. Further, the mass isotopologue distribution of metabolites known to be present in multiple compartments did not match distributions observed in EVs, indicating that EVs can package metabolites from mitochondrial compartments in addition to cytosolic compartments. Given these observations, and the incomplete knowledge of metabolite packaging in EVs, Exo-MFA lets the user define which intracellular compartments can contribute to EV cargo.

dividing yield of EVs (in protein or particle number) by the time over which the EVs were collected.

5. Load the 'Main_ExoMFA_source.m' script in MATLAB (see Note 17) and run until all the steps are completed.
6. Exo-MFA is based on 13-carbon metabolic flux analysis (13C-MFA) that utilizes Isotopomer Mapping Matrices (IMM) method to model 13-carbon atom transitions. The model is assumed to be at isotopic steady-state and is solved for intracellular fluxes, v , and mass isotopomer distributions, y .
7. Exo-MFA provides results for intracellular fluxes of source cells as well as packaging fluxes that represent metabolites packaged into EVs. The confidence intervals are also estimated by the script and published in an Excel file with the flux results. The MATLAB script also provides a file labeled 'exo_IDV' that is used as an input for estimating intracellular fluxes in recipient cells.
8. The empirically measured data for *recipient* cells is compiled in an input Excel file, for which the template can be downloaded from supplemental files. There should be a separate input file for each time point at which the recipient cells were sampled.
9. For *recipient* cells, enter measured extracellular fluxes in the first column of the sheet 'flux', followed by the standard deviation and name of the metabolite (as referred to in the model file). Enter the mass isotopologue distribution data for intracellular metabolites and intra-EV metabolites in the first column of the sheet 'MID', followed by the standard deviation of each measurement, name of the metabolite and the carbon chain. These are the measurements obtained in Section 39. In the sheet 'exo', list the metabolite names (without denoting the compartment) that are detected in the EV cargo in the first column, followed by the compartments in the recipient cells, which are possible recipients of those metabolites (see Note 18). The sheet labeled 'exo_IDV' should contain the data as published in the output file in step 3.11.7.
10. Load the 'Main_ExoMFA_recipient.m' script in MATLAB and run until all the steps are completed. Repeat this step for every time point at which recipient cells were sampled.
11. Exo-MFA provides results for intracellular fluxes of recipient cells as well as cargo release fluxes that represent EV metabolites being internalized by cells. The confidence intervals are also estimated by the script and publishes an Excel

¹⁷ExoMFA was developed with MATLAB 2016a and Artelys Knitro Solver 10.0. Using earlier or later versions of these software may lead to errors due to incompatibility.

¹⁸Internalization is assumed to be time-dependent due to dependence on extracellular concentration of EVs and the transient nature of the nutrient-deprived recipient cells. The cargo of EVs is consistent throughout the process of packaging, transport and internalization. Release of cargo into recipient cells is slightly more complex than packaging, since (i) not all metabolites are utilized in the same way, and (ii) intracellular metabolic fluxes are not at steady-state. Therefore, the EV cargo is categorized according to its utilization, i.e., (i) central carbon metabolites that are incorporated directly into central carbon metabolism which are either in the cytosol or mitochondria, and (ii) essential amino acids that are incorporated only into biomass.

file with the flux results. The Exo-MFA algorithm also estimates an additional parameter, $u(t)$, which represents the rate of EV internalization (see Note 18).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

Empirical and computational workflow for Exo-MFA. Recipient cells are cultured in stable-isotope labeled medium to produce EVs with isotope-labeled metabolite cargo. Upon isolation EVs are isolated and a fraction of them are introduced into culture medium for recipient cells. Metabolic analysis is performed on intracellular extracts from source and recipient cells, EVs, and spent culture medium using GC-MS. All empirical data is compiled as inputs to the Exo-MFA software to quantify intracellular fluxes in source and recipients cells as well as metabolic fluxes carried by EVs from source to recipient cells.

Figure 2:

(A) Depleted FBS is produced by ultracentrifugation of commercial FBS. The resulting solution can be used to supplement culture medium spiked with ^{13}C stable-isotope tracers to produce the stable-isotope labeled medium for production of EVs. (B) After culturing cells in EV-deprived stable-isotope tracer medium, EVs can be isolated via differential ultracentrifugation. After a series of increasing centrifugations, the purified EV pellet is finally collected at the bottom of the centrifuge tube where it can be resuspended and used for further analyses.

Figure 3:

(A) Source cells are cultured with stable-isotope labeled medium (e.g. ^{13}C -Glucose and ^{13}C -Glutamine). Spent medium with secreted EVs is collected and divided into two batches: the first batch is reserved for formulating culture medium for recipient cells and the second batch is analyzed using a GC-MS. (B) EVs carrying stable-isotope labeled metabolite cargo are introduced into culture medium for recipient cells. Cells are incubated in this medium and sampled at multiple time points within 24 hours in order to capture the dynamic metabolic contribution of EV metabolites. Both spent culture medium and intracellular extracts are analyzed using the GC-MS