

Supplementary information

Hyperpolarized NMR of plant and cancer cell extracts at natural abundance

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1. Details of experimental methods

1.1 Preparation of tomato extracts

Plant samples. Tomato (*Solanum lycopersicum* L. Moneymaker variety) plants were grown in a glass greenhouse in the South-West of France under commercially optimal conditions as described previously [1]. The fruits were harvested at 28 days post anthesis. They were green and had attained approximately half of their final fruit size. The tomato sample was made up of 4–5 fruits harvested on different plants. For each fruit, a quarter of the fruit pericarp from the equatorial zone was immediately frozen in liquid nitrogen. The fruit pericarp sample was ground in liquid nitrogen and stored at -80 °C until freeze-drying.

Extract preparation. Two extracts were prepared from the tomato fruit powder, one for conventional NMR and another for D-DNP-NMR analysis. Each extract was prepared from 20 mg lyophilized powder. The metabolite extraction was performed manually in two steps. After weighing 20 mg of the powder, 2 mL of EtOH/water (80/20, v/v) solution were added to each sample in a 13 mL screwed poly-ethylene tube, then the mixture was checked and put in a hot water bath at 80 °C for 15 min and finally placed in a centrifuge at 30,000 *g* for 10 min. The supernatant was transferred into another 13 mL poly-ethylene tube. The pellet was submitted to another extraction step with 2 mL EtOH/water (50/50, v/v). The two supernatants were combined and evaporated to dryness using a vacuum evaporator (SpeedVac Savant, Asheville, USA). For the dry extract assigned to conventional NMR analysis, ethylene diamine tetraacetic acid (EDTA, 2 mM final concentration) disodium salt was added to avoid paramagnetic effects on citrate and malate detection in ¹H NMR spectra. EDTA was not added in the dry extract intended for the D-DNP-NMR analysis to avoid the addition of salts that could potentially affect the glass formation when freezing the sample. A control sample was also prepared following the same steps as above, but without biological material.

Sample preparation for NMR analysis. For conventional NMR analysis, one of the dry extracts was solubilized in 700 µL D₂O, then transferred to a 5 mm NMR tube. For D-DNP-NMR, the second dry extract, as well as a control sample, were dissolved in 200 µL of H₂O/D₂O/Glycerol-d₈ (2:3:5) doped with 25 mM TEMPOL.

1.2 Preparation of breast cancer cell line extracts

Chemicals for cell cultures. Culture media Dulbecco's Modified Eagle Medium (D-MEM), antibiotics (penicillin-streptomycin), Fetal Bovine Serum (FBS), trypsin and Dulbecco's Phosphate Buffered Saline (D-PBS) were purchased from Gibco® by life Technologies (www.lifetechnologies.com). Glucose, glutamine, sodium pyruvate, phenol red, L-glutamine (¹⁵N₂, 98%) and D-glucose (U-¹³C₆, 99%) were purchased from Sigma-Aldrich (www.sigmaaldrich.com). Chloroform (grade Rectapur) and methanol (HPLC grade VWR) were purchased from VWR International (www.vwr.com). Deuterium oxide (D₂O; 99.9%D) was obtained from EURISO-TOP (www.eurisotop.com).

Protocol for cell cultures. Two cell cultures were prepared, one at natural abundance and another in enriched media. For the natural abundance culture, the following compounds were added to D-MEM: 7.5 mg red phenol, 55 mg pyruvate sodium, 290 mg glutamine, 2500 mg glucose. For the enriched culture, the following compounds were added to D-MEM: 7.5 mg red phenol, 55 mg pyruvate sodium, 217.5 mg glutamine, 72.5 mg ¹⁵N-glutamine, 1875 mg glucose, 625 mg ¹³C-glucose. The cell growth procedure was identical for the two cultures. Cells from the SKBR3 line were grown as monolayer cultures (56 x 10⁶ cells/175 cm² flask in two identical flasks) in D-MEM supplemented with 10% of FBS, 1% of 10,000 units/ml penicillin–10,000 units/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. When cells were confluent at 90 %, the culture medium was removed from the culture flask (175 cm²) and the cells were washed twice with D-PBS. Afterwards, cells were quenched using 5 ml of methanol and 1 ml MilliQ H₂O as described by Teng et al. [2] and detached from the culture flask using a cell lifter. For each cell culture, the content of the two flasks was finally pooled.

Extraction and extract preparation. Two extracts were prepared from isotopically enriched and natural abundance cultures, respectively. The suspensions (6×2 ml) were sonicated with a sonicator probe (Vibra cell™) for 3×5 s. The extraction was performed on cells in 27 mL of a MeOH/CHCl₃/H₂O solution (10:10:7), according to the procedure described in Ref. [3] to extract intracellular metabolites. 10 mL of cold CHCl₃ and 5 mL of ultra-pure cold water were then added and the mixture was vortexed for 15 s and kept on ice for 15 min. After

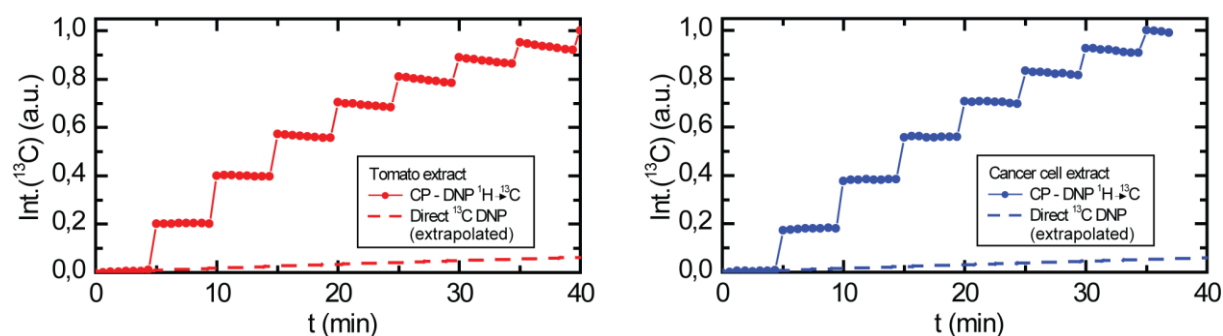
centrifugation (15 min, 4500 g, 4°C), the upper methanol/water phase was kept for analysis. The samples were dried by evaporation under a gentle flow of N₂ gas at 40 °C and were used for NMR analysis. The aqueous phase contained water-soluble low-molecular weight endogenous metabolites, while non-polar metabolites such as lipids were in the organic phase. Proteins and other biological macromolecules were precipitated by the addition of methanol and chloroform and trapped in the layer between the aqueous and organic phases.

Sample preparation for NMR analysis. The isotopically enriched extract was partitioned into two equal aliquots: one was dissolved in 700 µL D₂O and transferred to a 5 mm NMR tube. The other one was dissolved in 200 µL of H₂O/D₂O/Glycerol-d₈ (2:3:5) doped with 25 mM TEMPOL. The natural abundance sample analyzed by D-DNP-NMR was prepared in similar fashion

1.3 Dynamic Nuclear Polarization

DNP was performed at 1.2 K and 6.7 T in a home-built polarizer by applying microwave irradiation at $f_{\mu w} = 188.3$ GHz and $P_{\mu w} = 100$ mW with frequency modulation of amplitude $\Delta f_{\mu w} = 100$ MHz and modulation frequency $f_{mod} = 10$ kHz. ¹H → ¹³C cross polarization (CP) contacts were established at intervals of 5 min with 30 W applied to ¹H and 45 W to ¹³C (radio frequency amplitudes of 40 kHz on both channels). After 30 min polarization, the samples were dissolved with 5 mL of D₂O (preheated to T = 450 K at P = 1.0 MPa), transferred to a 500 MHz Bruker Avance spectrometer through a 1.5 mm inner diameter PTFE tube in a 0.8 T magnetic tunnel, pressurized with helium gas at 0.6 MPa. The injection was finally performed in into a 5 mm NMR tube. The complete dissolution process took about 10 s.

Figure S1 shows how the use of ¹H → ¹³C cross polarization (CP) yields faster and higher polarization than direct ¹³C polarization. Without CP, the polarization does not build-up properly because the very low ¹³C concentration in such samples prevents proper spin diffusion. This feature was already reported in previous publications, but is exacerbated in these low concentrated samples.



Supplementary Figure S1. CP-DNP build-up curves measured in the DNP polarizer for metabolic extracts of tomato (left, red dots) and cancer cells (right, blue dots). The dashed lines show the extrapolated direct ¹³C DNP build-up curve (which has not been measured beyond the first CP contact in this study, but has been recorded in other samples).

1.4 NMR experiments

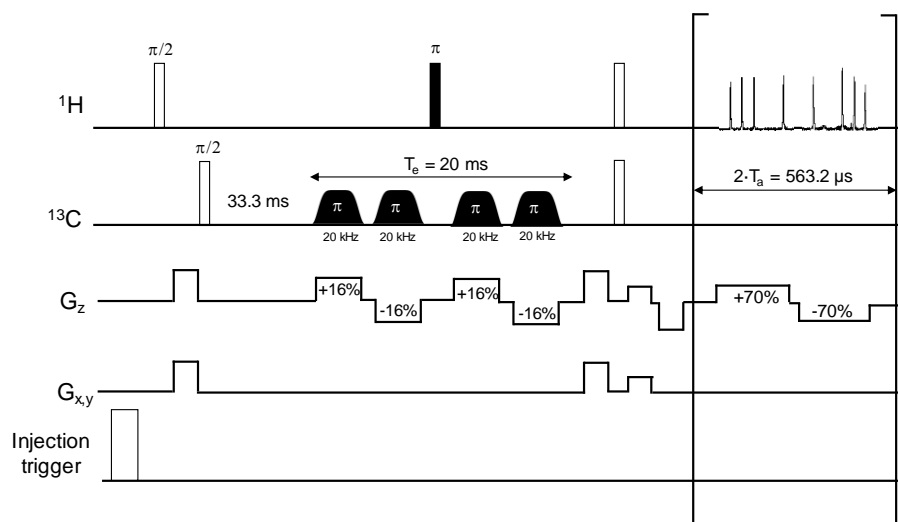
Spectrometers. All D-DNP experiments (Figs. 1b-c, Figs. 2b-c) as well as the conventional ¹H-¹³C HMBC spectrum (Fig. 2a) were recorded at 298 K on a 500 MHz Bruker Avance spectrometer equipped with an inverse cryogenic probe with triple axis gradients. The conventional 1D ¹³C experiment (Fig. 1a) was performed on a 700 MHz Bruker Avance III HD equipped with an inverse cryogenic probe with z-gradients.

1D ¹³C experiments. The conventional 1D ¹³C spectrum (Fig. 1a) was recorded in 11h 43 min (1024 scans) with an inverse-gated decoupling pulse sequence, with carefully calibrated 90° pulses, a 40 s recovery delay and a 1.2 s acquisition time. Waltz-16 ¹H decoupling was applied during the acquisition. The FID was multiplied by a decaying exponential (5 Hz line-broadening), then zero-filled to 128 k data points, Fourier transformed and baseline corrected. The D-DNP ¹³C spectra (Fig. 1b-c) were recorded in a single scan with an inverse-gated decoupling pulse sequence, with 30° pulses applied every 7.7 s (recovery time = 5 s, acquisition time = 2.7 s). This

procedure makes it possible to remove the first spectrum recorded immediately after injection in case its lineshapes would be affected by inhomogeneities, although this was not the case here. The successive FIDs were recorded in a pseudo-2D fashion, multiplied by a decaying exponential (5 Hz), zero-filled to 128 k data points, Fourier transformed and baseline corrected. Then the first 6 spectra (those contain most of the signal) were added to yield the spectra shown in Fig. 1b and c.

Conventional ^1H - ^{13}C HMBC experiments. The HMBC spectrum of Fig. 2a was recorded with a commercially available pulse sequence (hmbcgpndqf), including a gradient-based coherence selection but without decoupling during acquisition. The delay for the evolution under long-range couplings was set to 71.4 ms, corresponding to an average coupling constant of 7 Hz. FIDs were recorded with 4096 points and an acquisition time of 0.4 s. 1024 increments were recorded with 16 scans, leading to an experimental duration of 13 h 42 min.

Single-scan ^1H - ^{13}C HMBC experiments. The single-scan experiments in Figs. 2b and c were recorded with the pulse sequence of Figure S1, similar to the one proposed by Mishkovsky and Frydman [4], consisting in a direct excitation of the hyperpolarized ^{13}C followed by spatial-encoding and by a polarization transfer based on long-range couplings followed by echo-planar detection. Spatial-encoding was performed with a constant-time [5] spatial encoding scheme, with four 5 ms smoothed chirp ^{13}C pulses swept over a 20 kHz range and separated by a hard 180° ^1H pulse. The amplitude of the encoding gradients was adapted to obtain a frequency dispersion equivalent to the frequency sweep of the pulses, corresponding to 16% of the maximum gradient strength available. During the acquisition, 128 pairs of bipolar gradient pulses were applied (70% of maximum strength, 261.6 μs each, separated by a 20 μs delay). The delay for the evolution of long range couplings was set to 33.3 ms, corresponding to an average coupling constant of 15 Hz, in order to maximize the sensitivity with respect to J-modulation, due to the constant-time character of this experiment. Conventional coherence-selection gradients were also used. The spectrum was processed using a home-written routine in Matlab, including an optimized Gaussian apodization in the spatially-encoded dimension [6] and a sine-bell apodization in the FT dimension.



Supplementary Figure S2. Single-scan ^1H - ^{13}C HMBC experiment adapted for D-DNP experiments.

2. References for supporting information

- [1] B. Biais, C. Bénard, B. Beauvoit, S. Colombié, D. Prodhomme, G. Ménard, S. Bernillon, B. Gehl, H. Gautier, P. Ballias, J.-P. Mazat, L. Sweetlove, M. Génard, Y. Gibon, Remarkable Reproducibility of Enzyme Activity Profiles in Tomato Fruits Grown under Contrasting Environments Provides a Roadmap for Studies of Fruit Metabolism, *Plant Physiol.*, 164 (2014) 1204-1221.
- [2] Q. Teng, W. Huang, T.W. Collette, D.R. Ekman, C. Tan, A direct cell quenching method for cell-culture based metabolomics, *Metabolomics*, 5 (2009) 199-208.
- [3] O. Beckonert, H.C. Keun, T.M.D. Ebbels, J. Bundy, E. Holmes, J.C. Lindon, J.K. Nicholson, Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts, *Nat. Protocols*, 2 (2007) 2692-2703.

[4] M. Mishkovsky, L. Frydman, Progress in Hyperpolarized Ultrafast 2D NMR Spectroscopy, *ChemPhysChem*, 9 (2008) 2340-2348.

[5] P. Pelupessy, Adiabatic single scan two-dimensional NMR spectroscopy, *J. Am. Chem. Soc.*, 125 (2003) 12345-12350.

[6] P. Giraudeau, S. Akoka, Sensitivity and lineshape improvement in ultrafast 2D NMR by optimized apodization in the spatially encoded dimension, *Magn. Reson. Chem.*, 49 (2011) 307-313.