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Supporting Information

Enhanced ^{13}C NMR Detects Extended Reaction Networks in Living Cells

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Materials and Methods

Materials

Commercial dry yeast was used for evaluating the information content that is accessible using sensitivity-optimized ^{13}C D-DNP NMR experiments. The commercial dry yeast was suspended in YNB medium to final OD values (OD^{600}) of 45, 3 or 1. For in cell ^{13}C NMR observations, 0.2 ml cell suspension was transferred to a 5 mm NMR Shigemi sample tube in a 500 MHz Bruker spectrometer that was thermally equilibrated to 310 K. Authentic samples of glutamate, alanine, 3-phosphoglycerate, dihydroxyacetonephosphate, and pyruvate were obtained from Sigma Aldrich. These reference compounds were dissolved at 2-4 mg in 600 μl of 30 mM MES buffer (pH 6.5 in D_2O). ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC on an 800 MHz Bruker Avance III HD instrument equipped with a 5 mm TCI cryoprobe and a SampleJet sample changer. Assignments validated the assignment of glycolytic intermediates, and allowed the tentative assignment of newly observed signals as belonging to glutamate, which is the most abundant individual metabolite most model organisms.[S1]

The isotope-enriched metabolic substrates [U-^{13}C, U-^{2}H] glucose and [1-^{13}C,1-$^{2}H_2$]-glucose) were purchased from Sigma Aldrich (Andover, MA, USA).

Dynamic Nuclear Polarisation of hexoses

Hyperpolarization was performed as previously described.[S2] Briefly, solid state dynamic nuclear polarization of hexose substrate was conducted using trityl radical OX063 (27 mM; Oxford Instruments, Abingdon, UK) and gadoteridol (1.5 mM; Bracco Imaging, Italy). These final substrate samples contained 50 μmol hexose in 19 mg of aqueous polarization medium containing trityl radical OX063 and gadoteridol, and were placed in a sample cup for hyperpolarization. Samples were flash-frozen in liquid helium. The polarization transfer was conducted by DNP at 1.2 K, using microwave irradiation at 93.89 GHz with 100 mW in a magnetic field of 3.35 T for one hour. Solid-state polarization buildup was monitored to validate that solid-state polarizations above 30% were obtained. Samples were dissolved with heated MES buffer (5 ml of 30 mM, pH 5.65), thus yielding liquid hyperpolarized samples with a final substrate concentration of 11 mM hexose. Hyperpolarized substrates (0.33 ml) were manually injected into 0.2 mL of the cell suspension placed inside a 500 MHz Bruker spectrometer to yield a final concentration of 6.9 mM hyperpolarized glucose.

In vivo D-DNP NMR

All spectra were recorded on a Avance Neo 500 MHz NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a 5 mm DCH CryoProbe and a 11.7 T UltraShield magnet. The intracellular biochemical reactions were followed by a series of ^{13}C NMR spectra with a nominal pulse angle of 10º (enhancements indicating 12º), for excitation in pseudo-2D spectra using a receiver gain of 10. A ^{13}C NMR spectrum of 11264 complex data points (sampling the ^{13}C FID for 344.7 ms) was recorded every 0.5 s. As data acquisition was started prior to substrate injection, experimental dead-time upon substrate feeding was minimized. Alternatively, a single 1D ^{13}C NMR spectrum of 11264 complex data (sampling the ^{13}C FID for 344.7 ms) employing a EBURP shaped pulse covering the quaternary carbon region was created in the ShapeTool of

Topspin. The pulse was centered around an offset of 180 ppm was applied after defined times (6, 10, 20, 30, 60 seconds) post injection of hyperpolarized [U-^{13}C, U-^{2}H] glucose. All spectra were processed with extensive zero filling and an exponential line broadening of 10 Hz and were integrated in Topspin 4.07 (Bruker). Alternative acquisition schemes of hyperpolarization can be envisioned, but were not implemented. Detecting the hyperpolarized signal as a sum of two or three 1D ^{13}C experiments rather than a single 90º pulse experiment could allow for the diffusion of hyperpolarized signal into the active detected volume, thus possibly increasing the sensitivity further. Such detection schemes could conduct and sum two experiments with readout pulses of 45º and 90º, or three experiments with readout pulses of 35.36º, 45º and 90º.

Rapid injection In Vivo ^{1}H-^{13}C HSQC NMR

Glycolysis was followed with a time resolution of 2 min using narrow sweep width ^{1}H-^{13}C HSQC spectra acquired with 30% non-uniform sampling and reduced spectral width (80 ppm) in the ^{13}C dimension. An 11 mM [U-^{13}C] glucose solution in MES buffer was heated to 60 °C in a water bath. After rapid injection into the cell suspension through a room temperature capillary, this pre-warming ensured a temperature of 30 ºC in the NMR tube (determined using the linear dependency of the ^{1}H chemical shift of methanol relative to water in the temperature range from 298 – 313 K). These data support the assignments of the single scan D-DNP ^{13}C NMR experiments on the one minute time scale, by showing sizeable influx of ^{13}C label into amino acids (e.g. alanine) and citric acid cycle substrates (e.g. malate) in the 2-10 min time window.

Figures:

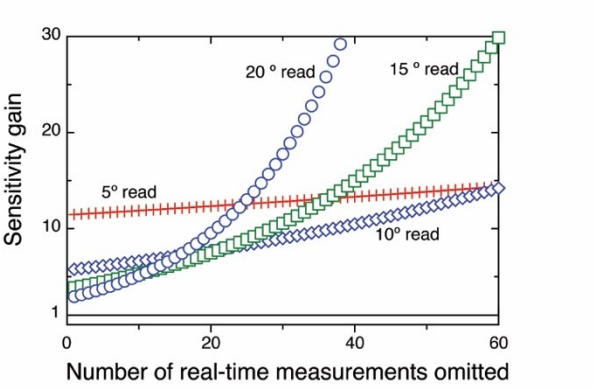

Figure S1. Sensitivity gains for the approach displayed in main text Figure 1B relative to real-time kinetic experiments, as a function of omitted real-time measurements and the pulse angle. Enhancements for n omitted pulses are calculated as $\left(sin\theta \times cos^{n}\theta\right)^{-1}$.

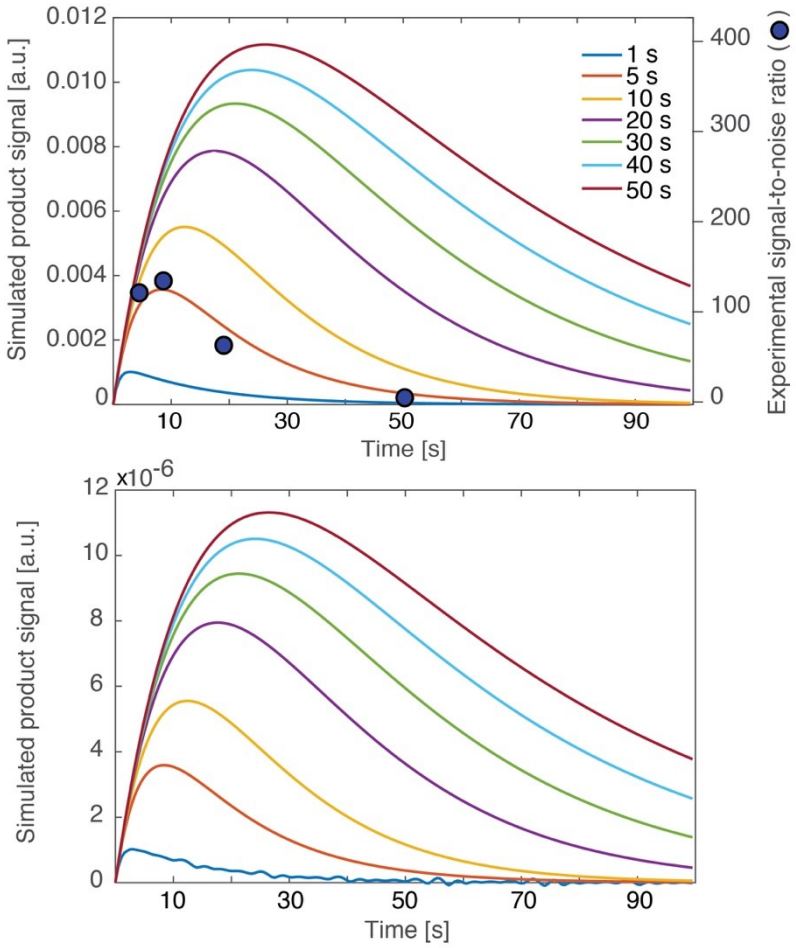

Figure S2. Simulation of expected product signal as a function of reaction time (x-axis) and product T_1 (different curves) in hyperpolarized NMR experiments. Curves simulate a substrate T_1 of 15 s ([U-^{13}C,^{2}H] glucose) and approximate the conversion as a first order reaction to product. The time, where maximum product signal intensity is observed, depends on the product T_1, but not on the reaction rate: the simulation in the bottom assumes a 1000fold smaller reaction rate than the simulation on top. Experimental signal-to-noise values for intracellular pyruvate acquired after 6, 10, 20 and 50 seconds are displayed as filled circles in the top figure. The simulations and results indicate that kinetic bottlenecks and relative changes in intracellular activity may be identified by relative changes in signal ratios at optimal detection times that depend on the substrate and product T_1. Simulations were conducted as described in [S3].

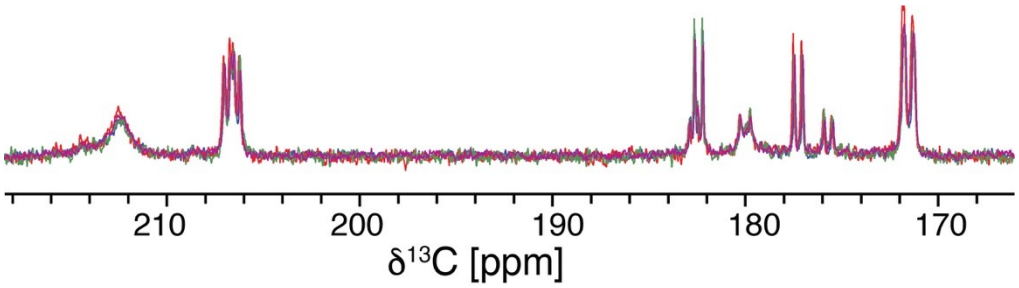

Figure S3. Repetition of four experiments with spectra acquired by a 90º pulse 20 seconds after injection of hyperpolarized glucose. The discontinuous assay proposed herein depends on reproducible measurements, which is found to be valid. A relative standard deviation below 8% for signal areas of in cell assays was determined by repetition in quadruplicate.

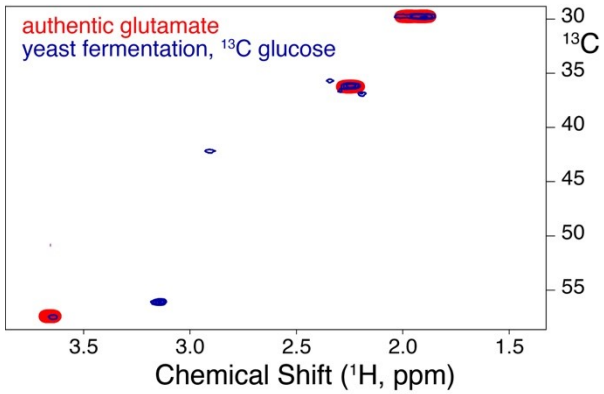

Figure S4. 800 MHz ^{1}H-^{13}C HSQC-NMR spectra of authentic glutamate and post-reaction material of a yeast fermentation, validating the presenece of NMR-detectable glutamate in the yeast fermentation.

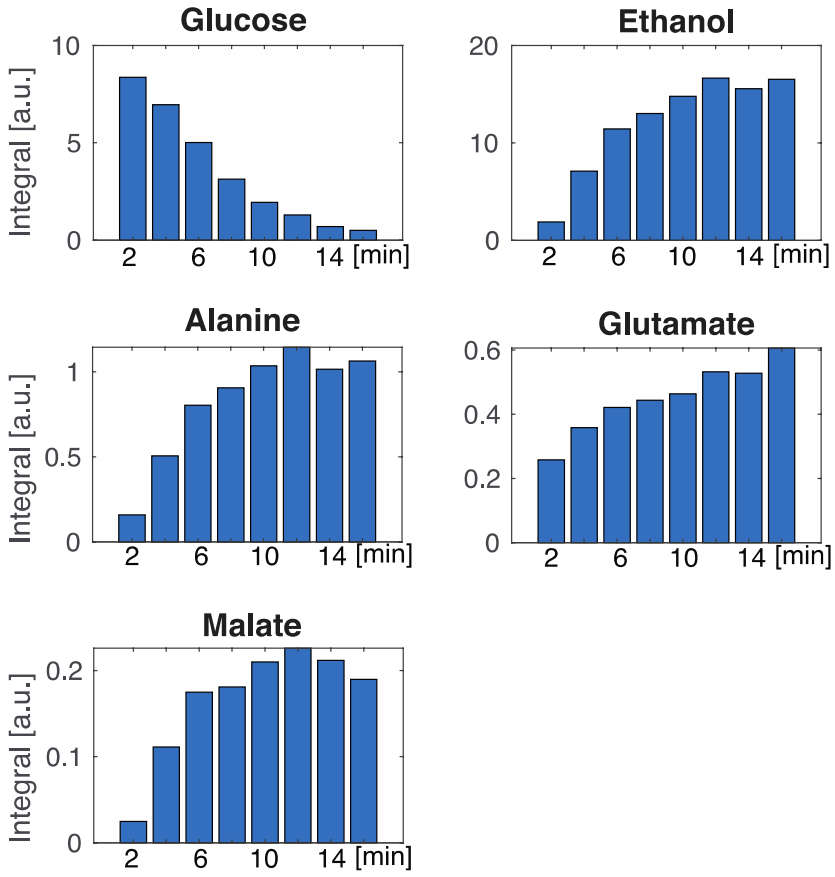

Figure S5. Glycolysis was followed with a series of ^{1}H-^{13}C HSQC spectra providing a time resolution of 2 min. A representative signal for each identified metabolite was integrated. These data support the assignments in the D-DNP NMR experiments.

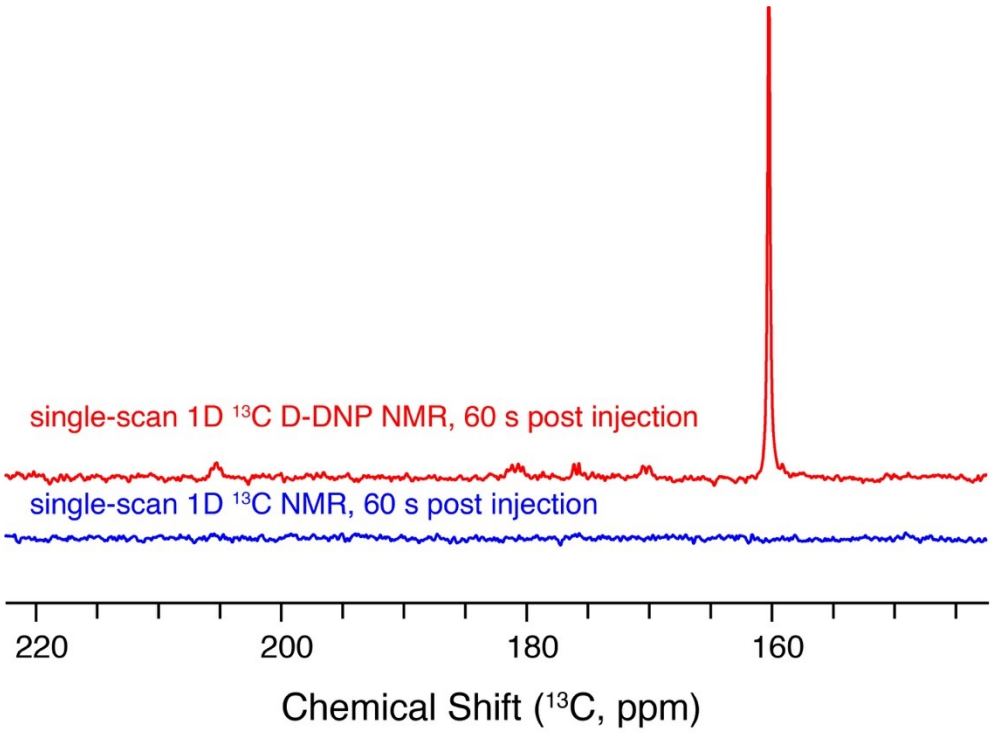

Figure S6. Main metabolite signals observable on the ~1 min time scale (including pyruvate, glutamate, alanine) are consistent with the most abundant metabolites in living yeast and are not observable without D-DNP. Kinetic in-cell experiments likewise show no metabolite signals beyond CO_2/HCO_3^- after 20 seconds of reaction (see main text Figure 2).

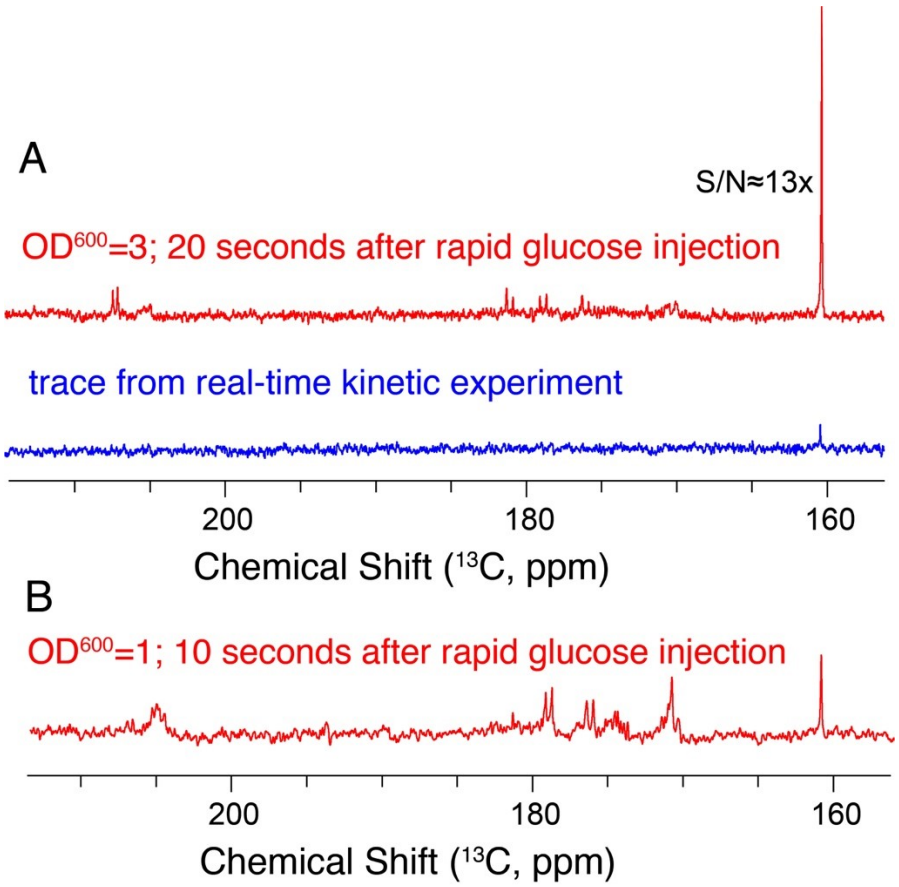

Figure S7. (A) Comparison of metabolite labelling as detected with optimized detection (red) and a kinetic experiment in a yeast cell suspension of OD^{600}=3 after 20 seconds of label influx. Metabolite signals are detectable only in the optimized assay at biologically common cell densities. (B) Metabolite labelling as detected with optimized detection in a yeast cell suspension of OD^{600}=1 after 10 seconds of label influx.

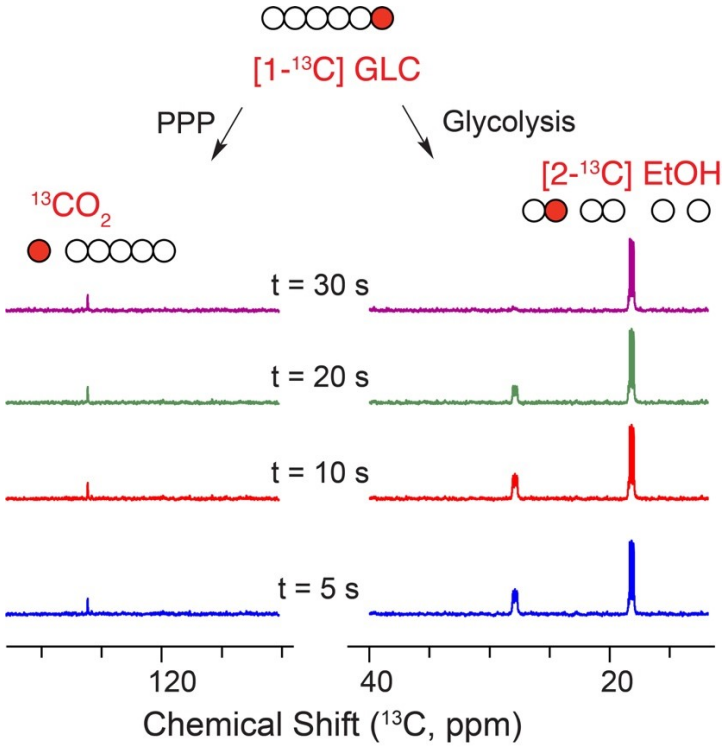

Figure S8. Experiments using hyperpolarized [1-^{13}C,^{2}H] glucose to distinguish between pentose phosphate pathway and glycolytic flux with D-DNP. The pentose phosphate pathway had been considered inaccessible to D-DNP NMR assays in yeast. We find the substrate to enter the pentose phosphate pathway and glycolysis at ratios 6.1%/93.9%, consistent with estimates from literature. The observations support that previously inaccesible metabolite levels can be assessed by sensitivity optimized discontinuous assays.

References:

S1: J. O. Park, L. B. Tanner, M. H. Wei, D. B. Khana, T. B. Jacobson, Z. Zhang, S. A. Rubin, S. H.-J. Li, M. B. Higgins, D. M. Stevenson, D. Amador-Noguez and J. D. Rabinowitz, *Nature Chemical Biology*, 2019, **15**, 1001-1008.

S2: S. Meier, M. Karlsson, P. R. Jensen, M. H. Lerche and J. Ø. Duus, *Molecular BioSystems*, 2011, **7**, 2834-2836.

S3: G. Pagès and P.W. Kuchel, *Magnetic Resonance Insights*, 2013, **6**, MRI.S11084.