Introduction
Elevated glycolytic rates have long been recognized as a feature of cancer. Following this aberrant metabolism in living systems with high temporal resolution is important in the understanding of cancer, and could be of valuable help in the targeted design of therapeutic treatments. $^{13}$C NMR is uniquely suited to following such metabolic processes in living systems; however the low polarization level of $^{13}$C nuclei requires long measurement times. The recent development of novel dynamic nuclear hyperpolarization (DNP)-based techniques to enhance liquid-state signals by ~10000x, enables us to take a fresh look at such metabolic processes with unmatched sensitivities and temporal resolution. We have previously reported a perfusion-injection system that allows hyperpolarized metabolic measurements to be performed on living perfused cells; in this study we apply this system to investigate Glucose metabolism.

Materials and Methods
Cell Culture: T47D (clone 11) human breast cancer cells were cultured in RPMI medium 1640 supplemented with 10% FCS (Biological Industries), 5 mL of L-glutamine, 0.4 mL of insulin, and 0.1% combined antibiotics (Bio-Lab). For the NMR perfusion studies, cells (>3 x 10^6) were seeded on 0.5 mL of Biosilon polystyrene beads (160–300 μm, Nunc) in silanized glass vials. Three hours later they were transferred to bacteriological dishes for cultivation, changing medium every second day, as well as on the day before the experiment. After 5–7 days of culture, cells on beads were transferred into a 10-mm test tube and connected to a perfusion-injection system described previously (2) for their NMR observation. For the radioactive transport studies T47D cells were plated on 6 wells tissue culture plates (Costar) and allowed to grow for 5 days. Incubation medium was exchanged the night preceding the experiment. Before the experiment, the media was aspirated and replaced with low-calcium Krebs-Ringer phosphate buffer containing 1 ¾ bovine serum albumin (KRP-BSA) and incubated at 37°C for 30 min to deplete intracellular glucose. The cultures were washed twice with KRP-BSA at room temperature for 5 minutes. The assay was performed by incubation of each well with 1 ml KRP-BSA buffer containing increasing concentrations of $^{13}$C 2-deoxy-D-glucose, covered by KRP-BSA at 37°C to a constant temperature for 5 minutes. At the end of incubation, radioactive medium was aspirated, and transport was stopped by addition of ice-cold KRP-BSA. Wells were washed for an additional three times with ice-cold PBS, followed by addition of ice-cold SDS (0.1 %). Resulting cell lysate was mixed with scintillation fluid for β-counting. Radioactive content was determined in liquid scintillation counter. Cells from two wells out of each plate were taken to determine protein concentration (Bradford). Initial rates (v) at varying glucose concentrations were calculated by linear fitting of the measurement taken at 3 time points (0, 1, and 3 minutes).

Hyperpolarization: A 4 M solution of U-$^{13}$C-D-D-Glucose (Cambridge Isotopes), 15 mM OX063 Trityl (GE Healthcare) and 0.5 mM of Ga-DOTA, was prepared in D.O. An aliquot of this sample was inserted into a Hypersense® 3.35-T low-temperature polarizer and irradiated at 1.5 K for executing DNP. After ~90 min of microwave irradiation, the sample was dissolved in 4 mL of phosphate buffer solution; this solution was heated to 180 °C, pressurized to 10 bar, and flushed into the perfusion–injection system within 4–5 sec. NMR Experiments: Spectra were recorded on an 11.7-T Varian spectrometer using a 10-mm broadband probe. These included $^{31}$P data recorded to monitor cell viability, and $^{13}$C spectra recorded immediately after injection of the hyperpolarized glucose solution; in the metabolism measurements a frequency selective pulse was used every 8 sec to better preserve the hyperpolarization.

Data Analysis: The $^{13}$C and $^{31}$P spectra obtained were processed using custom-written Matlab routines.

Results
The self-glassing 4M U-$^{13}$C-d$_1$ Glucose solution undergoes efficient hyperpolarization, with signal enhancement of approximately 10,000x observed in the liquid state. In order to maximize the lactate signal while preserving the Glucose polarization, a frequency selective pulse was used in which the frequency range of 120-220 ppm was pulsed at 90° while the glucose region was pulsed at ~1°. Upon injection to perfused T47D breast cancer cells, the decay of the glucose peaks can be observed in the range of 60-100 ppm, while the lactate peak can be observed growing at 183.5 ppm. (Figure 1a) Summing spectra from several injections reveals two additional metabolic peaks: one at 212.6 ppm, which can be tentatively assigned to Dihydroxyacetone Phosphate, and a broad peak at 179.2 PPM. (Figure 1b). Preliminary results show that the rate of conversion of Glucose to Lactate in T47D cells shows concentration dependence. Comparing this data to the initial rate of Glucose transport, calculated using $^{13}$C-2-deoxy-D-Glucose, the same concentration dependence is observed (Figure 1c). The latter study indicated a combined Michaelis –Menten and diffusion mechanism for glucose transport in these cells with K_m= 3.6±1.7 mM, V_{max}=6.6±2.5 pmol/min/mg and k(diffusion) = 1.46±0.06 pmol/mg/min/mM. This unprecedented ability to non-invasively measure in real-time the initial rate and labeling of glycolytic products and intermediate pools may provide new understanding of the altered metabolism of cancer and provide biomarkers that their metabolic kinetics may serve for diagnostic, prognostic and therapy response assessments.

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Figure 1: (a) $^{13}$C Spectra acquired after injection of 26mM of hyperpolarized U-$^{13}$C-d$_7$-D-
Glucose to perfused T47D breast cancer cells using a frequency selective pulse. (b) Sum of $^{13}$C spectra from four injections, revealing additional products (c) The graph on the left shows the initial rate of $^{13}$C-2-deoxy-Glucose transport as calculated by the zero-trans method and fit to a model with an enzyme facilitated and diffusive element. On the right this fit is compared to the rate of conversion of hyperpolarized Glucose→Lactate with the red line showing the fit from the radioactive results.