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Supplemental Information

Profiling Single Cancer Cells with Volatolomics Approach

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Supplemental Information

TRANSPARENT METHODS:

Reagents: Phosphate buffer saline pH 7.2 (PBS) and RPMI-1640 were purchased from Sigma Aldrich (St. Louis, MO, USA). Trypsin EDTA (0.25%), Penicillin and Streptomycin and Fetal Bovine Serum (FBS) were purchased from Biological industries, Israel. Two ml glass head space vials and magnetic crimp cap were purchased from CSI analytical innovations, Israel. T-75 flasks were purchased from Thermo scientific, Israel. All other chemicals of analytical grade were purchased from Sigma, USA. Internal stranded Mix (EPA-542) purchased from SUPELCO, Bellefonte, PA.

Cell lines: Three different human lung cancers and one lung normal cell lines were used for these experiments: A549, H1299, H1975 and BEAS-2B respectively. All the cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and all are derived from lung epithelial cell type.

Cell culture and Sample preparation: A549, H1299, H1975 and BEAS-2B (normal lung) cell lines were maintained in RPMI 1640 medium. In addition 10% fetal bovine serum and 1% penicillin and streptomycin were added to the RPMI. The cells were grown to 40-60% confluent monolayer in the 75 cm_2 culture flask under standard conditions at 37°C and 5% CO_2. After 15-24 h, remove the medium and washed twice with pre-warmed medium without FBS and FBS-free medium was added to the flask and incubated for 15-20 h for starvation, then the cells were harvested using 0.25% trypsin-EDTA and the cell suspension was transferred into centrifuge tubes to prepare a series of 10 fold dilutions. Then, 1µl of proper dilution was seeded (single cell) in 2 ml glass vial (flat bottom headspace vial) and the presence of a single cell was confirmed by microscopy. Thereafter, 500µl 0.5% FBS growth medium was added to each vial and the samples transferred for incubation. Seven biological replicates of each cell type were prepared for GC-MS analysis. Prior GC-MS analysis, the vials were sealed with magnetic crimp caps for 2 h (37°C and 5% CO_2) to boost the accumulation of species released by the cells and to block the gas exchange with the ambient air. For VOC measurement, all cell lines and control medium were incubated for T_0, T_{24} and T_{48} h. After incubation, sample and control medium (without cells) vials were immediately transferred for GC-MS analysis. Bulk cell samples were prepared similarly with some adjustments. $5*10^4$ cells were seeded in 2 mL glass vial and incubated for 24 and 48 h. After 24 h incubation period, no dead cells were observed in any of the cell lines. After 48 h, some floating cells were observed and the culture medium had turned red (all cell lines). Thus, the culture

conditions of the 24 and 48 h incubation periods ensured that the release of VOCs into the medium was mostly due to living cells (Figures S4 and S5).

Gas Chromatography-Mass Spectrometry (GC-MS) analysis: GC-MS analyses was performed using an Agilent 7890B series GC system (Agilent, USA) connected to an Agilent 5977A mass selective detector (MSD) (Agilent, USA) equipped with an extractor EI source. The analytical column was a SLB-5ms capillary column (with 5% phenyl methyl siloxane; 30m in length; 0.25 mm in internal diameter; 1µm in thickness; from Sigma-Aldrich). Ultra-high purity (99.999%) helium was used as carrier gas (flow-rate 1ml/min). The GC was operated under the following temperature program: initially at 35°C, held for 10 min at 200°C, held at 240°C for 21min, ramped at 15°C min^{-1} to 260°C, and held at 260°C for 2 min, giving a total run time of 25.7 min.

In-Tube Extraction (ITEX) Method: In this study we used an ITEX connected to GC-MS system for headspace sampling and analysis. The ITEX presents a higher extraction performance particularly when connected via the PAL-type fully automated ITEX device with needle packing (CTC Analytics AG, Switzerland) (http://phytronix.com/wp-content/uploads/2015/03/ITEX-Brochure-Low-Res.pdf). This technique has been widely applied and evaluated in different studies for extraction of volatile profiles from different samples (Laaks et al., 2010; http://www.palsystem.com/fileadmin/public/ docs/ Downloads /Brochures/pal-itex-bro-lr.pdf). Extraction process is fully automated and performed dynamically by moving the plunger of the syringe up and down to pump the sample headspace through the sorbent bed. Then, a fixed volume of an inert gas is aspirated in to the syringe as desorption volume. Before desorption process, the external heater is rapidly heated up to the desorption temperature and the analytes are ejected into the GC injector. After the needle is withdrawn from the GC injection port, the extraction device is flushed with an inert gas and heated to prevent carryover and to condition the extraction for a sample (Jochmann et al., 2008; Kedziora-Koch et al., 2018). Prior to starting the cell line experiment method optimization was done using the standard solution. Four different extraction temperatures were tested: 40, 60, 70 and 80°C. Seven different extraction cycles (strokes) were evaluated: 50, 100, 210, 350, 500, 750 and 1000. In addition, different extraction and desorption flow rates (100, 1000 µl/s), and sample volumes (1000 and 1300 µl) were tested.

Briefly, VOC extraction work flow in this study, the sample vial was set on an automatic sampling system connected to the GC-MS (Auto-PAL-RSI 120 system). Automated ITEX applied a 1.3 ml headspace syringe with a Tenax TA-filled needle body. The analysts were extracted from sample headspace by dynamic extraction on to the absorbent. The needle body was surrounded by a heating unit, which is used for analyte desorption into the injection port of a GC-MS. The auto-sampler was equipped with a single magnet mixer (SMM) and a temperature-controlled tray holder. The samples were placed in the tray cooler at 25°C; after transfer to the SMM, the sample was heated and stirred at 500 rpm for 20 min to reach the extraction temperature of 80°C to establish equilibrium

distribution of the analytes between liquid and gas phase in the vial before extraction. The extraction volume of the gas phase was set to 1000µl and 750 extraction strokes (20 sec for each stroke) were used for the optimized method for each sample. The extraction flow-rate during extraction was set at 100µl/sec. After the extraction, the sample vial was moved back to the tray. Desorption was performed in the step 3, the ITEX trap was heated to 250°C with desorption flow rate of 1ml purge gas was used to desorb and purge the extracted VOC of the sample at a flow-rate of 10µl/sec into the hot injector. After desorption, the ITEX device was flushed with nitrogen gas at 260°C for 5 min was applied. Afterward, the plunger was moved down, and the temperature was set to 80°C, to prepare the trap for the next extraction. The whole process (including injection, trap cleaning, and extraction of the following sample) was completed within the runtime of the GC oven program with cooling about 5 h. An internal standard mixture (EPA-542) 1,4-Dichloro benzene-D4 was added (7ppb) along with test samples as well as control medium to ensure that the GC-MS was functioning effectively, and data was normalized accordingly. The test was based on examination of the retention time and peak shape of the solvents used in the calibration mixture. All experiments were repeated seven times and the results expressed as the mean ± standard deviation.

GC-MS data processing: The GC-MS chromatograms were analyzed using Mass Hunter qualitative (version B.07.00; Agilent Technologies, USA) analysis. The compounds were tentatively identified through spectral library match NIST 14 (National Institute of Standards and Technology, USA). Qualitative analysis involved the area under the curve values; subtracting relevant media only headspace controls values (collected during the same experiment conditions). All experiments were repeated seven times and the results expressed as the mean ± standard deviation. The features were screened using two criteria: relative standard deviation <20% and detection score frequency >90%.

Calibration: VOCs identification and concentration were determined through external standards and calibration curves. For each VOC, pure standards were purchased (Sigma-Aldrich, MI, USA). The reagents, stock solutions were made to a concentration of 1M by dissolving them in 1 ml methanol. Calibration solutions of 1, 10, 50, 100 and 150 ppb were prepared in Methanol. VOC standards were diluted and measured using the same methods for measuring samples. Standard curves were created based on the peak areas, which were obtained from Mass Hunter Qualitative analysis. The data were analyzed in triplicate. The concentrations and RSD of VOC in GC-MS for analysis of each cell line was calculated and are given in Table S1.

Cell viability assay: Trypan blue assay was used to determine viability in a time-dependent manner. Briefly, single cell/vial was seeded in a 2 ml glass vial and incubated for the desired time-points. After incubation, spent medium was removed from the 2 ml glass vial, and 50 µl 0.4% Trypan blue (Biological industries, Israel) and 50 µl medium were added (1:1) added before incubation for 2 min. The samples were washed gently with PBS and viability checked under the bright field microscopy. Blue staining indicated dead cells, whereas viable cells excluded the dye. In addition single cell viability adherence and proliferation (in cases) were observed, further supporting the viability of the single cells tested (Figures 1 and S4).

Statistical Analysis: Statistical significance was calculated by the Kruskal-Wallis test, which is a test to compare samples from two or more groups of independent observations (Silva et al., 2017). It is a one-way ANOVA and does not assume a normal population, unlike the one-way ANOVA. The Kruskal-Wallis test is a nonparametric version of the classic one-way ANOVA, and an extension of the Wilcoxon rank-sum test to more than two groups (Kleinbaum et al., 1998). The patterns of the significant VOCs were confirmed using SAS JMP, Verison.12.0 (SAS Institute, Cary, North Carolina, USA; 1989, 2005). Additionally, results are presented as mean values with SDs.

SUPPLEMENTARY FIGURES AND LEGENDS

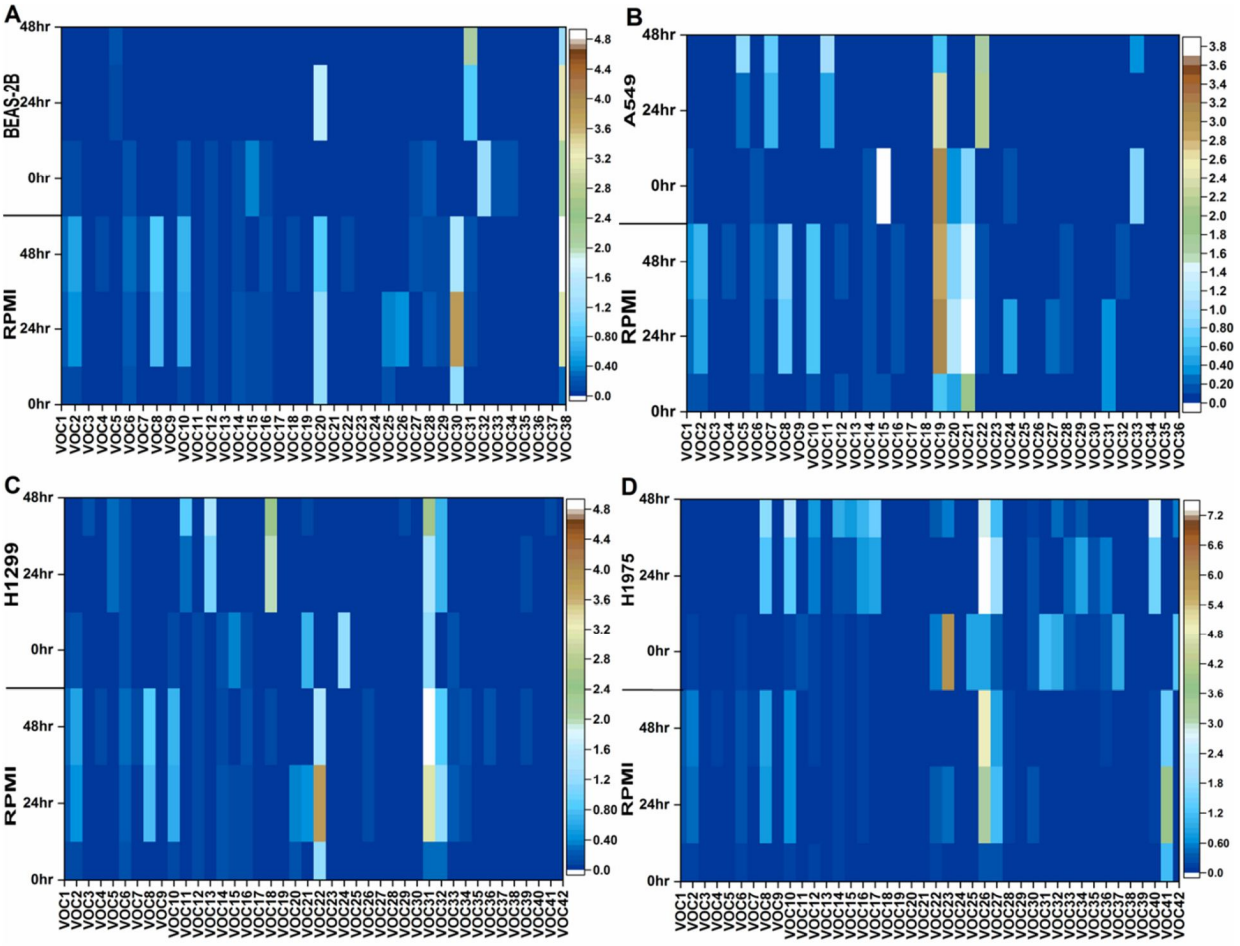

Figure S1: Profile patterns of VOCs detected at the single cell level of normal and cancer lung cell lines. Heat-map with all the selected VOCs from culture media and cells of ITEX-GC-MS. Color coding shows the abundance of each compound measured in the sample normalized to the maximum abundance calculated in all samples. Related to Figure 3. (A). Normal lung cell (BEAS-2B) (B). A549 lung cancer cell (C). H1299 lung cancer cell (D). H1975 lung cancer cells. (Note: VOC-Volatile organic compounds).

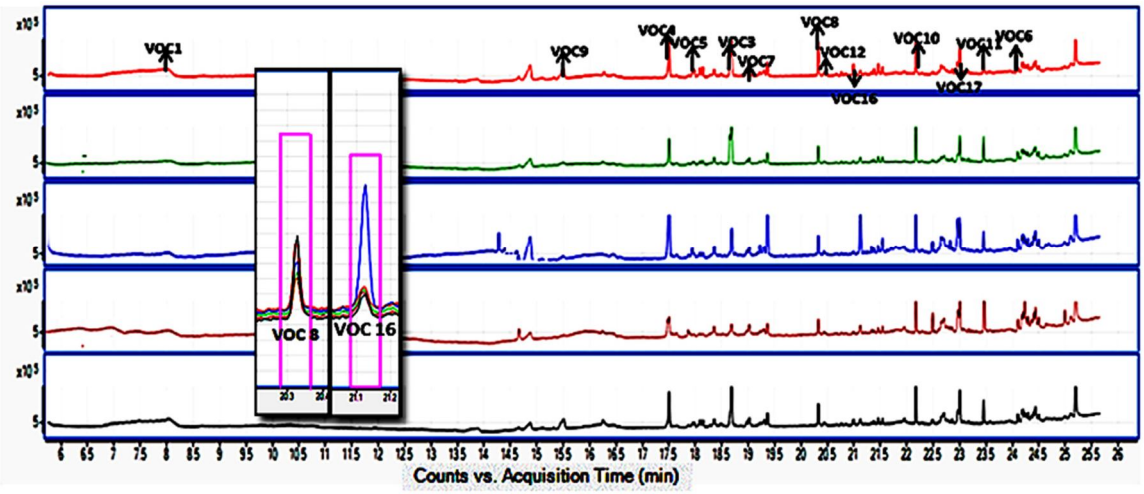

Figure S2: Representative total-ion-count (TIC) chromatograms of single cell samples and medium GC-MS chromatograms. Differences are shown in various peaks between the different cells. Related to Figure 3. Note: Cell free medium-Red; BEAS-2B-Green; H1299-Blue; H1975-Brown; A549-Black. Insert present a zoom-in example of two VOCs that presented significant differences as seen by differences in peak area.

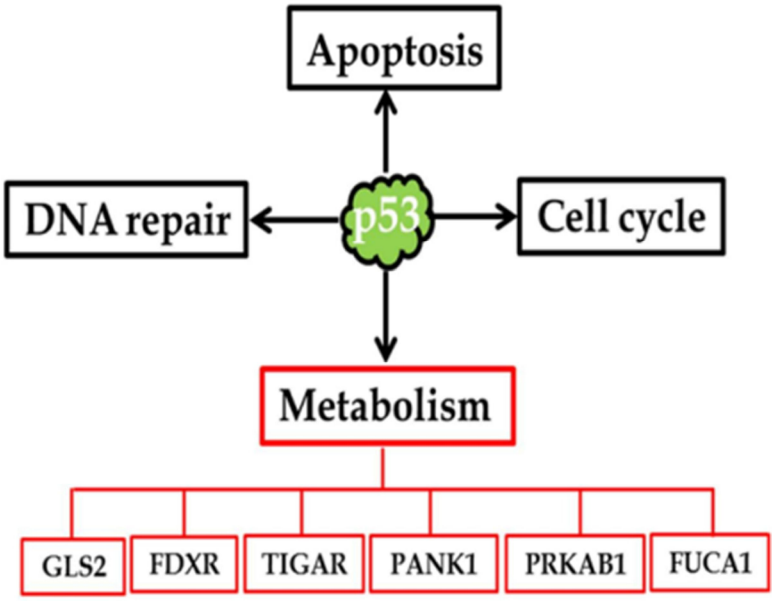

Figure S3:Tp53 directly activates target genes that mediate several functions. Proteins encoded by p53 target genes function in multiple processes that include cell cycle arrest, DNA repair, apoptosis and metabolism regulation. Related to Figure 5.

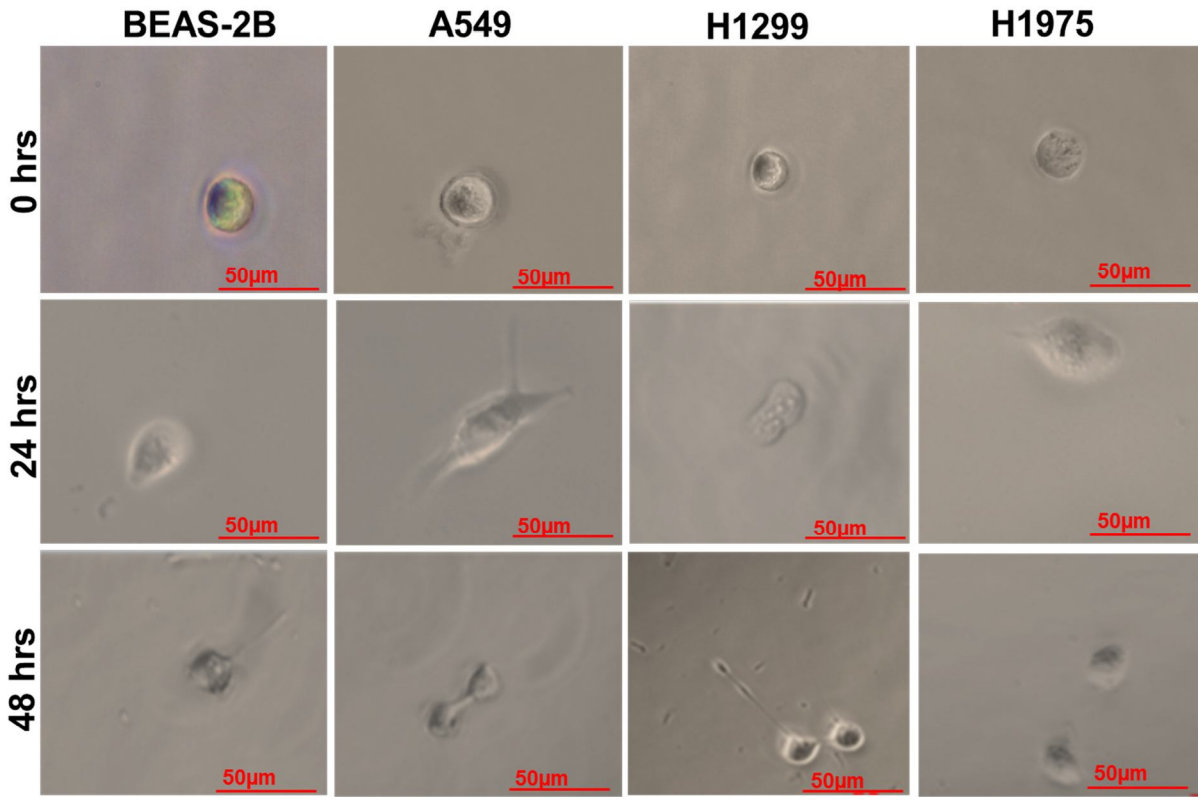

Figure S4: Single cells culture in regular medium: Trypan blue staining. Optical microscopic images of the BEAS-2B, A549, H1299 and H1975 cells. The cells were stained using trypan blue dye after T0, T24 and T48 hours incubation to examine cell damage. All single cells adhered to the vial bottom (×10 magnified). In each picture the scale bar indicate is 50µm. Related to Figure 1.

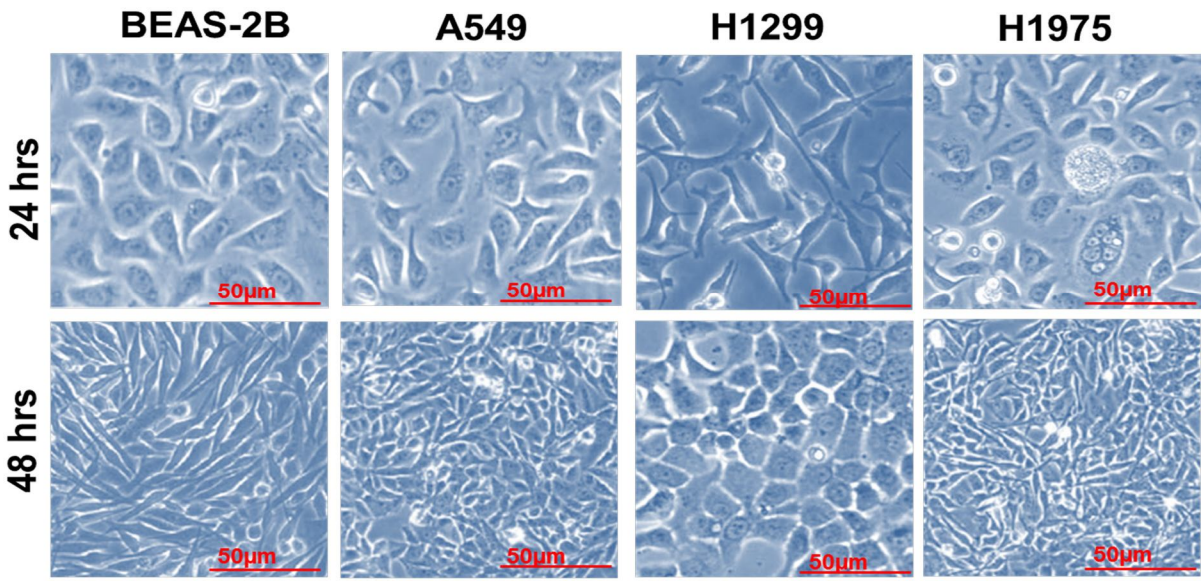

Figure S5: Bulk cells culture in regular medium: Trypan blue staining. Optical microscopic images of the BEAS-2B, A549, H1299 and H1975 cells. The cells were stained using trypan blue dye after T24 and T48 h incubation to examine cell damage. In each picture the scale bar indicate is 50µm. Related to Figure 1.

SUPPLEMENTARY BIBLIOGRAPHY

Jochmann, M.A., Yuan, X., Schilling, B., Schmidt, T.C. (2008). In-tube extraction for enrichment of volatile organic hydrocarbons from aqueous samples. J. Chromatogr. A 2, 96-105.

Kedziora Koch. K, and Wasiak W. (2018). Needle-based extraction techniques with protected sorbent as powerful sample preparation tools to gas chromatographic analysis: Trends in application. J. Chromatogr. A 31,1-18.

Kleinbaum, D., Kupper, L., Muller, A., Nizam, K. (1998). Applied Regression Analysis and Other Multivariable Methods Pacific Grove (CA): Brooks/ Cole Publishing Company.

Laaks J, Jochmann M.A, Schilling, B, Schmidt T.C. (2010). In-tube extraction of volatile organic compounds from aqueous samples: an economical alternative to purge and trap enrichment. Anal. Chem 15, 82,7641-7648.

Palsystem (2015). ITEX Dynamic Headspace Powerful Sample Enrichment for GC. http://phytronix.com /wp- content/uploads/2015/03/ ITEX-Brochure-Low-Res.pdf.

Palsystem (2010). ITEX-2 Option Prep and Load Platform High Sensitivity Enrichment Technique for Gas Chromatography. http://www.palsystem.com/fileadmin/public/docs/Downloads/ Brochures/pal-itex-bro-lr.pdf.