Profiling Single Cancer Cells with Volatolomics Approach

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HIGHLIGHTS
Measurement of VOCs was achieved at the single-cell level
Genetic changes influence the emitted volatiles of single and bulk cancer cells
Single-cell VOC analysis measures population heterogeneity in initial stage of tumors
Volatolomics research can promote non-invasive, simple, and cost-effective diagnostics

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SUMMARY

Single-cell analysis is a rapidly evolving approach to characterize molecular information at the individual cell level. Here, we present a new approach with the potential to overcome several key challenges facing the currently available techniques. The approach is based on the identification of volatile organic compounds (VOCs), viz., organic compounds having relatively high vapor pressure, emitted to the cell’s headspace. This concept is demonstrated using lung cancer cells with various p53 genetic status and normal lung cells. The VOCs were analyzed by gas chromatography combined with mass spectrometry. Among hundreds of detected compounds, 18 VOCs showed significant changes in their concentration levels in tumor cells versus control. The composition of these VOCs was found to depend, also, on the sub-molecular structure of the p53 genetic status. Analyzing the VOCs offers a complementary way of querying the molecular mechanisms of cancer as well as of developing new generation(s) of biomedical approaches for personalized screening and diagnosis.

INTRODUCTION

A vital challenge of biology is to understand how individual cells process information and respond to perturbations. Cells may appear morphologically identical but are heterogeneous and made up of individual cells that can differ significantly (Hou et al., 2016; Templer and Ces, 2008). These differences can have important consequences for the health and function of the entire population. Therefore, single-cell analysis that allows the definition of the cell (bio)chemical profile is of critical importance for in-depth study of diseases and drug development (Schmidt and Efferth, 2016; Heath et al., 2016; Broza et al., 2015).

Currently, there are several “omics” approaches employing single-cell analysis: genomics, proteomics, and transcriptomics (Wang and Bodovitz, 2010; Zenobi, 2013). For example, tumors’ heterogeneous nature is well established and is of great importance due to the option of deducing disease development data from genomic heterogeneity and revealing original insights about the impact and function of cancer stem cells (Di Carlo et al., 2012). Another example, single-cell resolution techniques has been used to track population subgroups for identifying the therapeutic potential of various drugs (Di Carlo et al., 2012). Although significant advances have been achieved with these approaches, several limitations restrict the fulfillment of the diagnostic and therapeutic applications. These limitations include, but are not confined to (Zenobi, 2013; Amantonico et al., 2010), the following: (1) proteomics and genomics require prior and accurate knowledge of specific genes or proteins and are exclusive to in vitro and in vivo trials, something that does not necessarily reflect real-life situations and (2) genomics and proteomics still suffer from high cost, low specificity, and complex analysis algorithms, which result in prolonged and cumbersome analysis (Rockwell, 1980; Wilkins et al., 1996; Chung et al., 2007; Khoo et al., 2016). Besides these limitations, there is also a need for a biomarker that provides systematic knowledge of the disease without the need to isolate and explore specific genes or proteins.

In this article, we present a new frontier for single-cell analysis. The approach is based on the isolation of individual cells by serial dilution approach and the analysis of their volatolomics profile, viz., the volatile organic compound (VOC) profiles emitted into their headspace (i.e., the gas environment trapped closely above the cell). VOCs are chemical compounds that have a low molecular weight and relatively high vapor pressure under room temperature conditions (Broza and Haick, 2013; Hakim et al., 2012; Nakhleh et al., 2017; Broza et al., 2018). An intriguing feature of the VOCs is their widespread partition coefficient in fat and air or blood (i.e., a coefficient designed to estimate the equilibrium concentration of VOCs in fat tissue and [lipophilic] cell membranes with respect to fat/blood), indicating their (hypothetical) participation in the signaling pathways of the cell (Barash et al., 2009; Haick et al., 2014).
Several studies have been conducted to investigate the VOCs emitted from bulk (in vitro) cancer cells, which include millions of cells (Filipak et al., 2008; Sponning et al., 2009; Amal et al., 2012; Chen et al., 2007; Davies et al., 2014; Peled et al., 2013). Nevertheless, bulk-based approaches may not provide insight into whether differences in VOC expression between samples (Jia et al., 2018; Silva et al., 2017) are driven by changes in cellular composition, changes in the underlying phenotype, or changes in the signaling pathways (Sellick et al., 2011; Yamamura et al., 2005). One of the issues in the field is the fact that tumors (or bulk analysis) are heterogeneous and thus could result in VOC profiles that present wider variations. Yet, each tumor starts with just one cell, and this is exactly the rational of examining just a few cells. One main reason for studying at the single-cell level is that it allows us to avoid population heterogeneity in late stages and precisely measure early initiation of tumors as well as the potential reasons, signal promoting tumorigenesis and even heterogeneity. In addition, ensemble measures do not provide insights into the stochastic nature of the VOC expression. Indeed, VOC single-cell analysis can provide solution in some parts compared with other single-cell methods (as described above), yet it also necessitates cell harvesting and analysis in a controlled in vitro setting and would benefit from future technological solutions to ease analysis. Intrigued by these challenges, we report on a VOC-based approach for single-cell analysis that has the potential to overcome several key challenges that face the currently available techniques in this field. This concept is demonstrated by lung cancer cells with various p53 genetic statuses that were isolated from A459: p53⁰⁰⁰, H1299: p53⁰, and H1975: p53M cell lines. Normal lung cells (BEAS-2B: p53W) that were isolated manually are used as a reference. Knowing a VOC profile at the single-cell level can also be useful in developing novel cancer diagnostic approaches. As the VOC profile reflects various events in the cells, the information contained in the cellular VOC profile may be useful for identifying the heterogeneity in cell population. It is also believed that volatile profiles deliver a more immediate and dynamic picture of the functionality of a cell. Hence, knowing VOC profile at the single-cell level can be very useful for developing novel cancer diagnostic approaches and can offer more understanding on the VOCs’ behavior (concentrations) linked with cancer states.

RESULTS AND DISCUSSION

VOCs from Single Cells Measured by GC-MS

In this study, we have identified VOC profiles from single cells that were isolated from lung cancer cell lines. As a reference, we have identified the single cancer cell profiles with normal lung cells (BEAS-2B) at the single-cell level. To examine the influence of the molecular profile of (isolated) single cell on the VOCs’ profile, we have compared between lung cancer cells that express different p53 statuses (A549, H1299, and H1975). As the (bio)chemical behavior of the cells change with time, we have also examined the VOCs’ profile at different time points: T₀, T₂₄, and T₄₈ hr. With regard to the time course, the single cell may or may not survive for long time; hence it is important to check cell viability at T₂₄ and T₄₈ hr, at which times cells were stained with trypan blue for 1 min and washed with PBS. Cell viability was checked by microscopy, and all cell lines had a viability of >95% (Figure 1).

The volatile profile of each cell line was determined by gas chromatography-mass spectroscopy connected with the ITEX device (ITEX-GC-MS; see Transparent Methods section for more details). The ITEX is a fully automated sample preparation technique that facilitates an autonomous desorption step. It has several advantages over other methods (e.g., Solid Phase Micro Extraction) for VOC detection, such as, a needle resistant to mechanical damage, protection of the extraction phase, long lifetime of the extraction phase, thermal desorption process independent of the GC injector temperature profile, reduced susceptibility to contamination, and reusability. The procedure of ITEX sampling evaluated for GC-MS analysis is presented in Figure 2. The VOCs arising from single cells were studied using ITEX-GC-MS at times, T₀, T₂₄, and T₄₈ hr. Normal lung cells and cell-free media were used as intra-experimental controls. By using the NIST14 mass spectral library, a total of 250 VOCs were identified in all samples with a library matching >90%. After deducting the VOCs found in the background samples, 36–42 different VOCs in each sample remained (Figures S1A–S1D and S2). Here we used glass vials for the main cell culture step as they present very limited release of volatile chemicals (plastic flasks release plasticizers that give extra peaks) that might interfere with analysis (Koyuturk et al., 2007; Zimmermann et al., 2007). It has to be noted that the length of the incubation time and supplementation of cell culture medium have an effect on the VOC profile at the single-cell level can be very useful for developing novel cancer diagnostic approaches and can offer more understanding on the VOCs behavior (concentrations) linked with cancer states.

VOC Profile Varies Temporally among Genetically Different Cells

We noticed only 18 VOCs that were statistically correlated with the different cell lines showing for each of them specific each pattern was time-dependent (Figure 3). The detected compounds belong to classes of alkanes, aldehydes, ketones, alcohols, hydrocarbons, and phenols derivatives (Table 1). From Figure 3, VOC 15 and VOC 17 were identified only in the H1975 cell line (p53-mutant) in all time points; VOC 11
was found in A549 (p53-null) and H1299 cells (p53-null) at T0, T24, and T48 hr. These VOCs were unique for
the examination of each cell line. VOCs 1, 2, 4, 5, 6–10, 12, 14, 16, and 17 were found to be common in both
lung cancer and normal cells, but significantly differed in the levels of peak area (p < 0.01).

Comparative analysis between the different cell lines with control medium at different incubation times used
MassHunter qualitative software. The concentration of the 18 VOCs was determined using a standard curve
that was prepared with analysis by GC-MS. Standard curves were induced using the peak area. Calibration
measurements were used to determine the limit of detection (LOD) under experimental conditions of our
instrument and average concentration levels of 15 VOCs; the remaining VOCs could not be acquired (undeca-
ne-3,8-dimethyl-; nonane-5-methyl-5-propyl-; heptane-2,2,4,6,6-pentamethyl-). Figure 4 and Table S1
summarize the average concentration levels as well as the calculated LOD of 15 VOCs in parts per billion
units. Significant changes were detected from all compounds after incubation times of T24 and T48 hr, but
no significant changes were observed at T0 hr (Table S2). The standard curve of each VOC indicated
good linearity in the range 1–150 ppb (R² = 0.98–0.99). The concentration of each VOC was then calculated
using the respective standard curves (Table S1). In some cases, lower parts per thousand levels were calcu-
lated by extrapolating beyond the LOD. The range of concentrations found here fit previous bulk studies
reporting on concentrations from a few parts per billion to thousands of parts per billion (Peled et al.,
2013; Filipiak et al., 2008, 2010; Sponring et al., 2009).

At T0 hr of the incubation period, there were no significant differences between control media
and cell lines. At T24 hr of incubation, there was a difference in the abundance of 14 VOCs in the
A549 cells compared with the control media. Of the 14 VOCs, 10 compounds were significantly
decreased (butanal-3-methyl-, butanal-2-methyl-, benzaldehyde, benzophenone, dodecane, tetradecane,
pentadecane, nonane-5-methyl-5-propyl-, dodecane-4,6-dimethyl-, 2,4-di-tetra-butylphenol) and four
VOCs (acetophenone, 1-hexanol-2-ethyl-, 2,4-dimethyl-1-heptene, and hexadecane) were marginally
increased. At T48 hr of incubation, the concentrations of the above-mentioned 10 VOCs were strongly
decreased in A549 compared with the medium control. On the other hand, the four compounds that
moderately increased at T24-hr incubation increased significantly further at T48-hr incubation (p < 0.01;
Table S3).

In the H1299 cell line, 16 VOCs were detected, although at T0 hr there were no significant changes. At T24 hr,
a significant change was recognized in their concentration compared with control media. Nine of the VOCs
(butanal-3-methyl-, butanal-2-methyl-, benzaldehyde, dodecane, 2,4-dimethyl-1-heptene, tetradecane,
pentadecane, nonane-5-methyl-5-propyl-, dodecane-4,6-dimethyl-) were significantly decreased. Five VOCs (nonanal, 1-hexanol-2-ethyl-, hexadecane, undecane-3,8-dimethyl-, 2,4-di-tetra-butylphenol) were slightly increased. At T48 hr, the concentrations of the nine VOCs had strongly decreased (to near 0) and those of the 5 VOCs were significantly increased (p<0.01) compared with the control medium (Table S4).

In H1975 cells, 14 VOCs were identified. At T0 hr, no significant changes in VOC levels were found in comparison with normal cells and the control media. At T24 hr, six VOCs (butanal-3-methyl-, butanal-2-methyl-, benzaldehyde, benzophenone, acetophenone, 2,4-di-tetra-butylphenol) had significantly decreased. Eight VOCs (dodecane, 2,4-dimethyl-1-heptene, tetradecane, pentadecane, nonane-5-methyl-5-propyl-, heptane-2,2,4,6,6-pentamethyl-, dodecane-4,6-dimethyl-, heptane-4-methyl-) had become significantly increased. At T48 hr, the six VOCs had decreased markedly and the eight VOCs had significantly increased compared with the medium (p<0.0001) (Table S5).

In normal lung cells, 13 VOCs were recognized, and at T0 hr no significant changes in them were found compared with the medium. At T24 hr, 13 VOCs had significant differences in their concentrations compared with the medium. Eleven VOCs (butanal-3-methyl-, butanal-2-methyl-, benzaldehyde, benzophenone, acetophenone, dodecane, tetradecane, pentadecane, nonane-5-methyl-5-propyl-, dodecane-4,6-dimethyl-, 2,4-di-tetra-butylphenol) were significantly decreased, and two VOCs (1-hexanol-2-ethyl-, 2,4-dimethyl-1-heptene) were significantly increased (p<0.01). At T48 hr, the concentrations of 11 VOCs had decreased radically and those of two VOCs had greatly increased compared with the medium (Table S6).

Butanal-3-methyl-, benzaldehyde, and butanal-2-methyl- were lower in cancerous and normal cell lines at T24 and T48 hr (Figures 5A–5C), which is consistent (in the increasing and decreasing) with previous studies with HepG2 cells (liver carcinoma cells) showing uptake of the aldehydes in cell cultures (Mochalski et al., 2013; Feinberg et al., 2017). Numerous reports have described lower concentrations of...
butanal-3-methyl- and butanal-2-methyl- compared with the medium and were thought to be consumed by cancer cells (Filipiak et al., 2008; Mochalski et al., 2013). Among other explanations, butanal-3-methyl-concentration decrease in cells could be due to impairment of oxidative phosphorylation in cancer cells or an increase in ALDH activity (ALDH1A1 and ALDH3A1) in lung cancer cells (Patel et al., 2008).

Comparison of VOC Profiles between Single and Bulk Cells

For comparison reasons, we performed a general bulk cell analysis of the same lung cancer cell lines. The VOCs arising from bulk cells at T24 and T48 hr were studied using the same ITEX-GC-MS methodology (see Figure 2 and Transparent Methods section for more details). The summary of the different VOCs identified by MassHunter qualitative analysis are listed in Table S7. The table also provides the direction change (increase or decrease) of the abundances among the A549, H1299, H1975, BEAS-2B, and control medium. Under our experimental conditions an average of 750 peaks per sample were detected by deconvolution analysis. The significantly increased or decreased peaks were selected and were tentatively identified by spectral library match using the NIST 14. Only VOCs with a library match score higher than 90% were considered. Validation or absolute concentrations were not determined for the bulk analysis, as it was not part of the main goal presented here. Results show that after 24-hr incubation period, there was a difference in the peak area of 23 compounds in all cells compared with the control, whereby 20 VOCs were found to be significantly increased and three compounds were found to be significantly decreased (p <0.05) (Table S7). After 48-hr incubation period, differences in the peak area of 39 VOCs between all cell lines and control medium were detected, whereby 31 VOCs were significantly increased and eight VOCs were significantly decreased (p < 0.05) (Table S7). Under our experimental conditions, the composition of the detected VOCs was different for each cell line and further varied according to the incubation period. These VOCs mostly consisted of ketones, hydrocarbons, and alcohols. Ten of the VOCs in the 24- and 48-hr incubation period were found to be common to both groups. Comparison between the bulk and single-cell levels showed 12 common VOCs (butanal-3-methyl-, nonanal, benzaldehyde, 1-hexanol, 2-ethyl-, benzophenone, acetonaphone, tetradecane, undecane-3,8-dimethyl-, nonane-5-methyl-5-propyl-, heptane-2,2,4,6,6-pentamethyl-, dodecane, 4,6-dimethyl-, 2,4-di-tetra-butylphenol) found in

Figure 3. Heatmap of the VOC
VOCs detected in ITEX-GC-MS of lung normal and cancer cell lines. Color coding shows the AUC of each compound measured in the sample normalized with STD AUC calculated in all samples. (Note: VOC1: butanal-3-methyl-; VOC2: butanal-2-methyl-; VOC3: nonanal; VOC4: benzaldehyde; VOC5: 1-hexanol-2-ethyl-; VOC6: benzophenone; VOC7: acetonaphone; VOC8: dodecane; VOC9: 2,4-dimethyl-1-heptene; VOC10: tetradecane; VOC11: hexadecane; VOC12: pentadecane; VOC13: undecane-3,8-dimethyl-; VOC14: nonane-5-methyl-5-propyl-; VOC15: heptane-2,2,4,6,6-pentamethyl-; VOC16: dodecane-4,6-dimethyl-; VOC17: heptane-4-methyl-; VOC18: 2,4-di-tetra-butylphenol).
examined cell lines from different time points. Although sample types produce different biomarker profiles, the fact that some compounds are actually similar is an encouraging outcome, strengthening our single-cell results.

Comparison of our results with previous bulk cell line studies provide some interesting data; whereas most VOCs found in bulk are different from those found significant in single cells Peled et al. showed that benzaldehyde levels were substantially lower in the headspace of bulk lung cancer cell lines compared with RPMI medium after incubation for ~68 hr (Peled et al., 2013). Our results here show that indeed RPMI medium levels were significantly higher after 48 hr than the lung cancer cells; moreover, the benzaldehyde concentration in our results were below the LOD (1 ppb), and this correlates as well with the estimation of Peled et al. (Peled et al., 2013). They showed that the average concentration of benzaldehyde for an estimated bulk 7 × 10^6 cells of H1975 (and related cells) is approximately 145 ppb. Based on their result we estimate the concentration in each single cell as 0.2 × 10^-3 ppb. This calculation is indeed lower than 1 ppb, although it still 2- to 3-fold lower than actually measured concentration. This comparison support previous data, but it also shows that bulk and single-cell studies bring different insights that might be influenced by the heterogeneity of bulk analysis. In the A549, benzaldehyde trends were similar between our single-cell results and those of Peled et al., whereas a recent study showed that benzaldehyde in A549 relatively increased compared with the medium (Jia et al., 2018). These differences stress the need to perform VOC evaluation on all levels, from the single-cell level, to a few cells, to bulk, and up to real-breadth studies.

Benzenophene significantly decreased in cells at all time points, except for H1299 cells, in which no significant change was seen (Figure 5D). However, bulk data presented slight increase between 24 and 48 hr. Previous studies showed that benzenophene levels have been found to be lower in A549 cells compared with cancer cell-free growth medium (Hanai et al., 2012). Takemoto et al. explained the take up of

| Chemical Group | Compound | CAS No. | m/z | Mean Retention Time ± 0.01 (min) | Coding |
|----------------|----------|---------|-----|---------------------------------|--------|
| Aldehydes      | Butanal-3-methyl- | 590-86-3 | 44  | 7.95                            | VOC1   |
|                | Butanal-2-methyl- | 96-17-3  | 57.1| 8.31                            | VOC2   |
|                | Nonanal      | 124-19-6 | 57  | 19.29                           | VOC3   |
|                | Benzaldehyde | 100-52-7 | 105 | 17.59                           | VOC4   |
| Alcohols       | 1-Hexanol, 2-ethyl | 104-76-7 | 57.1| 18.35                           | VOC5   |
| Ketones        | Benzenophene | 119-61-9 | 105 | 23.95                           | VOC6   |
|                | Acetophenone | 98-86-2  | 105 | 19.02                           | VOC7   |
| Hydrocarbons (straight chain) | Dodecane | 112-40-3 | 57.1| 20.32                           | VOC8   |
|                | 2,4-Dimethyl-1-heptane | 19549-87-2 | 70  | 15.36                           | VOC9   |
|                | Tetradecane | 629-59-4 | 57.1| 22.17                           | VOC10  |
|                | Hexadecane  | 544-76-3 | 57.1| 23.45                           | VOC11  |
|                | Pentadecane | 629-62-9 | 71.1| 20.76                           | VOC12  |
| Hydrocarbons (branched chain) | Undecane-3,8-dimethyl- | 17301-30-3 | 71.1| 21.2                            | VOC13  |
|                | Nonane-5-methyl-5-propyl- | 17312-75-3 | 71.1| 19.11                           | VOC14  |
|                | Heptane-2,2,4,6,6-pentamethyl- | 13475-82-6 | 57.1| 17.9                            | VOC15  |
|                | Dodecane-4,6-dimethyl- | 61141-72-8 | 71.1| 21                             | VOC16  |
|                | Heptane-4-methyl- | 589-53-7 | 43  | 13.62                           | VOC17  |
| Phenols        | 2,4-Di-tert-butylphenol | 96-76-4  | 191 | 23                              | VOC18  |

Table 1. Identification of VOCs from Investigated Human Lung Normal and Cancer Cell Lines by ITEX-GC-MS at Single-Cell Level
benzophenone by cancer cells via an increase in the metabolism rate as a result of high oxidation rate of fatty acids in cancer cells (Takemoto et al., 2002). Ketone bodies could be also associated with amino acid metabolism (Murray et al., 2006). Acetophenone has been identified in both cancerous and normal cell lines. The levels of this VOC were found to be significantly increased in A549 cells, but no significant changes were found in H1299, BEAS-2B, and H1975 cells compared with control medium (Figure 5E). This is consistent with previous reports showing an increase in the acetophenone level in A549 cells compared with the culture medium (O’Neill et al., 1988; Agmon et al., 2016).

2,4-Dimethyl-1-heptene significantly increased in all cell lines, although only slight changes were seen in H1299 cells at T24 and T48 (Figure 5F). 2,4-Dimethyl-1-heptane was previously detected in the headspace of lung cancer cells and normal cells (Filipiak et al., 2010). Hakim et al. found that 2,4-dimethyl-1-heptane could serve as a biomarker of lung cancer and suggested that it probably arises due to oxidative stress (Hakim et al., 2012). Hexadecane significantly increased in A549 (p53-wild) and H1299 (p53-null) cells compared with BEAS-2B (p53-null) and H1975 (p53-null) cells at all time points (Figure 5G). Interestingly, pentadecane significantly decreased in BEAS-2B, A459, and H1299, but in H1975 cells it increased markedly at all time points (Figure 5H). This compound might be a biomarker for breast cancer (Phillips et al., 2010), yet no documentation in relation to lung cancer has been found.

Nonanal and undecane-3,8-dimethyl were found only in H1299 cells (p53-null), and their level significantly increased in a time-dependent manner (Figures 5I and 5J). Haick and co-workers have reported a decrease in the concentration of nonanal in H774, H69, H187, and HS26 cells (Barash et al., 2012), but in our present study, nonanal levels increased in H1299 cells because these cells are p53 null-type. Bulk results showed increase at 24 hr, but a decrease after 48 hr. These differences can emphasize the changes occurring during growth progression that may cause some compounds to change between early stage (few cells) and late stage (millions of cells). Although no clear answer can be given based on current study, it might be related to heterogeneity changes of the bulk growth. Additional explanation for this increase may be the absence of the gene hampering metabolic regulation in the cell. It remains unclear how different forms of null-type p53 affect tumor metabolism. p53 regulates the metabolic pathways through its downstream targets (Figure S3) (Clendening et al., 2010). 1-Hexanol-2-ethyl-greatly increased in A549 cells at T24 and T48 hr compared with other cells (Figure 5K), but was not found in

Figure 4. Concentration Levels of VOCs Detected in Lung Normal and Cancer Cell Lines
Color coding shows the parts per billion levels of each compound concentration calculated in all samples. (Ash color indicates no compound). (Note: VOC1: butanal-3-methyl-; VOC2: butanal-2-methyl-; VOC3: nonanal; VOC4: benzaldehyde; VOC5: 1-hexanol-2-ethyl-; VOC6: benzophenone; VOC7: acetophenone; VOC8: dodecane; VOC9: 2,4-dimethyl-1-heptene-; VOC10: tetradecane; VOC11: hexadecane; VOC12: pentadecane; VOC16: dodecane-4,6-dimethyl-; VOC17: heptane-4-methyl-; VOC18: 2,4-di-tetra-butlyphenol).
of mutant p53 have different actions on tumor metabolism (Clendening et al., 2010; Liu et al., 2015). With
although wild-type p53 can regulate many other aspects of metabolism, it is unclear how different forms
of signaling, such as glucose starvation, nutritional deprivation, DNA damage, and oncogene activation.

To how p53 regulates different aspects of metabolism in different cell types in response to different stress
signals, the study reveals that all cell lines were consuming several compounds such as aldehydes and hydrocarbons, but fewer compounds from other chemical families. Decreased levels of aldehydes can be reasonably explained by the higher activity of aldehyde dehydrogenase in cancer cells. Nevertheless, the overall reasons for differences in VOC release or consumption between the examined cell lines are currently unknown but may result from phenotypic or genotypic differences. The power of VOCs may allow a different niche in metabolome related to volatile markers and, thus, open a whole world of disease biomarkers, which may shed some light on the hidden pathways of molecular transduction. Significant single-cell volatolomic data will provide insights to permit the development and testing of hypotheses that might identify the fundamental biological mechanisms and address clinical issues in diagnostics and diseases. Nevertheless, current developments indicate a forthcoming model shift from analysis of volatolomics to the study of single-cell volatolomics, which will enhance other “omics” approaches on the path toward better combined systems biology of single cells. Finally, the reported approach may serve as enabler source for developing new generation(s) of biomedical approaches for personalized screening, diagnosis, and future screening of various diseases (Sanchez Mdel et al., 2012).

Figure 5. Comparison of VOCs in the Headspace of Culture Medium from Lung Cancer Cell Lines Compared with Normal Cell Line after 0-, 24-, and
48-hr Incubation

Average normalized area (n = 7) with SD. (A) Butanal-3-methyl-; (B) benzaldehyde; (C) butanal-2-methyl-; (D) benzophenone; (E) acetophenone; (F) 2,4-
dimethyl-1-heptene; (G) hexadecane; (H) pentadecane; (I) nonanal; (J) undecane-3,8-dimethyl-; (K) 1-hexanol-2-ethyl-; (L) dodecane; (M) tetradecane; (N)
onane-5-methyl-5-propyl-; (O) dodecane-4,6-dimethyl-; (P) heptane-2,2,4,6,6-pentamethyl-; (Q) heptane-4-methyl-; (R) 2,4-di-tert-butylphenol.

H1975 cells (p53-mut). Therefore, it could be considered a biomarker for lung cancer. Our bulk study also showed an increase after 24 hr but did not show significant change after 48 hr. This VOC was shown to increase in the headspace of cancer cells NCI-H2087 (113.87 ppb) and A459 compared with the medium in previous studies as well (Sponring et al., 2009; Chen et al., 2007). Sanchez et al. reported that 1-hexanol-2-ethyl- might be a biomarker in saliva for patients with lung cancer. These results indicate that the origin of this alcohol is probably from the metabolism of body fluids and of alkanes, which may be altered in different histological types of lung cancers (Sanchez Mdel et al., 2012).

Of the remaining VOCs, those in Figures 5L–5R were significantly increased only in the H1975 cell line (p53-mut) at all time points compared with other cells. H1975 cells are mutant-type p53, so this gene could contribute to the metabolic regulation; currently, however, the role of mutant-type p53 in tumor metabolism remains mostly unknown (Peled et al., 2013; Barash et al., 2015; Koyuturk et al., 2007). Interestingly, mutant-type p53 and wild-type p53 proteins often regulate the same cellular biological processes, but with opposite effects. For example, in metabolic regulation, wild-type p53 inhibits glycolysis, whereas mutant-type p53 promotes glycolysis through distinct mechanisms (Davies et al., 2014). Little seems to be known as to how p53 regulates different aspects of metabolism in different cell types in response to different stress signals, such as glucose starvation, nutritional deprivation, DNA damage, and oncogene activation. Although wild-type p53 can regulate many other aspects of metabolism, it is unclear how different forms of mutant p53 have different actions on tumor metabolism (Clendening et al., 2010; Liu et al., 2015). With that, clearly other existing differences, mutations within the human cell lines can influence some of these VOC changes and should be carefully addressed in the future.

This report provides preliminary evidence that single cells of (lung) cancer have unique (volatile) molecular prints; each cell sub-type, even if with only minor difference(s) in its genetic structure, has a unique volatile molecular print. Thus the presence of one cell would not screen out the others. The study reveals that all cell lines were consuming several compounds such as aldehydes and hydrocarbons, but fewer compounds from other chemical families. Decreased levels of aldehydes can be reasonably explained by the higher activity of aldehyde dehydrogenase in cancer cells. Nevertheless, the overall reasons for differences in VOC release or consumption between the examined cell lines are currently unknown but may result from phenotypic or genotypic differences. The power of VOCs may allow a different niche in metabolome related to volatile markers and, thus, open a whole world of disease biomarkers, which may shed some light on the hidden pathways of molecular transduction. Significant single-cell volatolomic data will provide insights to permit the development and testing of hypotheses that might identify the fundamental biological mechanisms and address clinical issues in diagnostics and diseases. Nevertheless, current developments indicate a forthcoming model shift from analysis of volatolomics to the study of single-cell volatolomics, which will enhance other “omics” approaches on the path toward better combined systems biology of single cells. Finally, the reported approach may serve as enabler source for developing new generation(s) of biomedical approaches for personalized screening, diagnosis, and future screening of various diseases in a non-invasive, inexpensive, and portable manner.

Limitations of Study

There were some limitations in the present study. First, here we fixed the background baseline of experiment by using the same culture media for all cells as done in many previous studies, thus BEAS-2B cells were not cultured in recommended media. This approach can ensure that the media per se is not the source of different compounds, something that cannot be done when using different media as previously published. Nevertheless, future studies should be performed in the recommended. Second, bulk data were calculated via peak area rather than via absolute concentration, as it was not the main goal here. Third, we have used only the ATCC-validated lung cancer cell lines in “p53 Hotspot Mutation Panel” dedicated for studying p53 molecular mechanisms. We refer to changes as related to the p53 difference because this is a major difference; however, other differences might be responsible for some of the changes in results and as such should be interpreted cautiously. Fourth, to isolate and grow single cells we have used serum-free media for slow proliferation. This might have also affected
the VOC profiles; however, this step was performed on all cell lines, thus ensuring the same baseline to all. Finally, VOC detection at the single-cell level remains technically challenging due to many fundamental limitations, including the rapid change of the VOCs, small sampling volumes, low amount of VOCs, and diverse range of volatiles present in the cell, along with the present inadequacies in the sensitivity of analytical instruments. Still, the technology used to analyze the volatiles from single cells can provide valuable insights into the biological interactions - something that genomics and transcriptomics might be unable to deliver. This will allow understanding the unique properties of cells, cell-to-cell communication, and cell-environment interactions.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Transparent Methods, five figures, and seven tables and can be found with this article online at https://doi.org/10.1016/j.isci.2018.12.008.

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AUTHOR CONTRIBUTIONS
M.S., Y.Y.B., and H.H. conceived and designed the experiments. M.S. and Y.Y.B. interpreted the data, prepared the figures and tables, and wrote the manuscript. M.S. performed the experiments and analyzed the data. H.H. and A.M. directed the research. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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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 cm² culture flask under standard conditions at 37°C and 5% CO₂. 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% CO₂) 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 T₀, T₂₄ and T₄₈ 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*10⁴ 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 1000 µl 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 100 µl/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 1 ml purge gas was used to desorb and purge the extracted VOC of the sample at a flow-rate of 10 µl/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, Version.12.0 (SAS Institute, Cary, North Carolina, USA; 1989, 2005). Additionally, results are presented as mean values with SDs.
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.
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