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Lessons from single cell transcriptome analysis of oxygen-sensing cells

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Abstract

The advent of single cell RNA-Sequencing (RNA-Seq) technology has enabled transcriptome profiling of individual cells. Comprehensive gene expression analysis at the single cell level has proven to be effective in characterizing the most fundamental aspects of cellular function and identity. This unbiased approach is revolutionary for small and/or heterogeneous tissues like oxygen-sensing cells in identifying key molecules. Here, we review the major methods of current single cell RNA-Seq technology. We discuss how this technology has advanced the understanding of oxygen-sensing glomus cells in the carotid body and helped uncover novel oxygen-sensing cells and mechanisms in the mice olfactory system. We conclude by providing our perspective on future single cell RNA-Seq research directed at oxygen-sensing cells.

Keywords

Next-generation sequencing; hypoxia; arterial chemoreceptor; carotid body glomus cell; olfactory sensory neuron type B cell

Introduction

Oxygen is the major substrate for oxidative phosphorylation, a highly efficient energy production pathway that enabled organisms to grow in size and complexity. As a tradeoff, vertebrates, especially mammals, become highly dependent on the provision of oxygen. Changes in environment, activities, cardiovascular or pulmonary functions can result in arterial oxygen tension fluctuations. To maintain oxygen homeostasis, specialized oxygen-sensing cells that monitor and correct undesirable deviations have developed. Located bilaterally at the carotid artery bifurcations, the carotid body (CB) is the predominant sensor for sensing and adjusting acute hypoxemia (Gonzalez, et al., 1994, Lopez-Barneo, et al., 2016, Prabhakar, 2013). This pair of neural crest-derived sensory organs is not only remarkably small but also complicated in structure. As an arterial chemoreceptor, the CB is highly vascularized and receives dense innervations. Two major cell types are present in the

CB, with neuron-like glomus cells enveloped by supporting sustentacular cells. The glomus cells can instantly depolarize and release neurotransmitters in response to even a moderate drop in oxygen tension, activating afferent nerve fibers that relay information to the brainstem to increase ventilation and sympathetic outflow (Kumar, 2009, Kumar and Prabhakar, 2012).

While the CB was discovered almost a century ago, much of the knowledge on glomus cell properties was characterized in the past few decades, thanks in part to techniques such as patch-clamp that permitted physiological experiments on individual glomus cells. Majority of these studies were physiology- or pharmacology-based and generated important discoveries that became the foundation for the membrane theory: CB glomus cells express oxygen-sensitive potassium channels and voltage-dependent calcium channels that cause depolarization and neurotransmitter release (Buckler and Vaughan-Jones, 1994, Duchen, et al., 1988, Lahiri, et al., 2006, Lopez-Barneo, et al., 1988, Shimoda and Polak, 2011, Urena, et al., 1994). However, these membrane channels alone do not suffice to explain the upstream oxygen-sensing process. Researchers are now also employing a genetic approach to study genes encoding candidate oxygen sensors by characterizing corresponding knockout mice. This trend has led to several impactful publications in the past few years, each illustrating different mechanisms of oxygen sensing. (Chang, et al., 2015, Fernandez-Aguera, et al., 2015, Peng, et al., 2010, Yuan, et al., 2015). Solely relying on physiology or pharmacology experiments offers limited new and unbiased information when selecting candidate genes, yet traditional biochemical or molecular experiments are difficult to perform on CB due to its small size and heterogeneity.

The advent of single cell RNA-Sequencing (RNA-Seq) technology provides a new avenue of opportunities towards understanding the transcriptome profile of CB glomus cells. By creating a list of genes abundantly and/or specifically expressed in these cells, it serves as a relatively unbiased resource for mining candidates of the oxygen-sensing apparatus. Similarly, this approach could also be applied to other oxygen-sensing cells, often existing in small quantity or are relatively inaccessible, such as the aortic body, the pulmonary arterial smooth muscle cells, the pulmonary neuroepithelial body, the neonatal adrenal medulla, and even an unexpected organ such as the olfactory epithelium. The purpose of this review is to highlight the basic concept of single cell RNA-Seq technology and its recent development. More importantly, we will discuss its recent applications to the field of oxygen-sensing cells to generate new insights and how it can be used in the future to answer additional questions.

Single cell RNA-Seq technology

Soon after the advent of next-generation sequencing technology, it was quickly adapted to profile single cell transcriptome by modifying previous single cell transcriptome amplification protocols used for single cell qPCR and microarray (Tang, et al., 2009). The single cell RNA-Seq approach circumvents the application limitation (small input RNA) posed by conventional RNA-Seq and carried over many of its advantages (Wang, et al., 2009). It offers nucleotide-resolution accuracy with high sensitivity and a wide dynamic range, allowing better quantification of mRNA transcripts, identifications of splice isoforms and allelic expression patterns. Without the need to predefine hybridization probes, it also

enables discovery of novel transcripts. The ability to profile the transcriptome of a single cell broke the bottleneck for many rare cell types. Many functionally important cell types are often located within complex structure or are scarcely available, such as specific subtypes of neurons or early embryonic cells. It was traditionally difficult to characterize the transcriptional features of such cells in a high-throughput manner or without contamination from nearby tissues. By applying single cell RNA-Seq to a wide variety of cells, we gained new knowledge on potential molecular players without being biased by a preformed hypothesis. Also, as more single cells are being sequenced, new revelations of previously unappreciated cellular heterogeneity are uncovered. On one hand, greater understanding on transcriptional regulation is building as we start to notice unexpected transcriptome variations among the same cell types, in part due to alternative splicing, random monoallelic expression, cell cycle, and stochastic bursts (Buettner, et al., 2015, Sasagawa, et al., 2013, Shalek, et al., 2013, Shalek, et al., 2014, Tang, et al., 2011). On the other hand, new subpopulations or even new functional cell types are emerging in diverse tissues (Chen, et al., 2017, Furlan, et al., 2016, Grun, et al., 2015, Muller and Diaz, 2017, Patel, et al., 2014, Saraiva, et al., 2015, Tirosh, et al., 2016, Villani, et al., 2017, Zeisel, et al., 2015b). Combined with newly derived bioinformatics algorithms, it is becoming possible to map out the evolving transcriptional programs or cellular states that govern key stages in development (Blakeley, et al., 2015, Fletcher, et al., 2017, Petropoulos, et al., 2016, Treutlein, et al., 2014, Xue, et al., 2013).

As the name suggests, single cell RNA-Seq requires isolation of individual cells (Fig. 1a). For rare cell types such as oxygen-sensing cells, this has been accomplished by picking dissociated single cells in suspension by mouth-pipetting or a micromanipulator (Omura and Mombaerts, 2015, Tang, et al., 2010, Usoskin, et al., 2015, Xue, et al., 2013, Zhou, et al., 2016). Cells can also be captured directly from tissues using laser micro-dissection (Luo, et al., 1999, Wang and Janes, 2013). More automated and high-throughput methods are becoming available to isolate single cells. Fluorescence-activated cell sorting (FACS) can sort large quantities of fluorescent-labeled single cells into multiwell plates, which is especially helpful for studying cells with known markers or for enriching certain cell types, but requires a larger amount of starting materials (Jaitin, et al., 2014, Saraiva, et al., 2015, Villani, et al., 2017). The latest trend in single cell isolation is the utilization of microfluidic technology that enables large parallel single cell isolations in nano- or pico-liter volumes using either microfluidic chambers, microdroplets or microwells. An automated microfluidic chamber system, the Fluidigm C1 integrated fluidic circuit, can capture up to 96 or 300 cells in one chip followed by in situ cDNA synthesis and amplification, greatly reducing time and labor (Pollen, et al., 2014, Treutlein, et al., 2016). But to isolate a massive number of single cells in parallel, microdroplet technology is gaining popularities as in theory unlimited numbers of single cells can be compartmentalized into sub-nanoliter lipid droplets. The small reaction volumes greatly reduce reagent cost, when combined with beads bearing cell-specific barcodes, it is now feasible to capture and amplify the transcriptomes of thousands of single cells in one tube using the Drop-Seq, inDrop, or Chromium GemCode technology (Klein, et al., 2015, Macosko, et al., 2015, Zheng, et al., 2017). This has facilitated commercial platforms, such as the Chromium Single Cell 3' Solution, that offer streamlined workflows covering the entire process from sample input to data analysis. The

standardization of single cell RNA-Seq will promote its adoptions to broader biological contexts. However, these methods require specialized supporting equipment and may face extensive optimization processes to avoid low-capture rates. For experiments constrained by cost and sample input, microwells that separate single cells by gravity can serve as an alternative (Fan, et al., 2015, Gierahn, et al., 2017).

Immediately following single cell isolation are cell lysis, cDNAs synthesis and amplification (Fig. 1b). After the addition of lysis buffer, primers containing a stretch of oligo(dT) sequence are used to selectively reverse transcribe polyadenylated RNAs. The synthesized cDNAs are further amplified by PCR (Islam, et al., 2011, Picelli, et al., 2013, Ramskold, et al., 2012, Sasagawa, et al., 2013, Tang, et al., 2009) or in vitro transcription (IVT) (Hashimshony, et al., 2016, Hashimshony, et al., 2012, Jaitin, et al., 2014). Based on such strategy, a variety of single cell RNA-Seq methods have been devised to accommodate the needs for different experimental aims, each with its own advantages and disadvantages. These have been extensively reviewed and systemically compared in experimental settings (Kolodziejczyk, et al., 2015, Saliba, et al., 2014, Svensson, et al., 2017, Ziegenhain, et al., 2017).

The major disadvantages of single cell RNA-Seq needs to be considered before analyzing any data (Table 1 summarizes a comparison between bulk RNA-Seq and single cell RNA-Seq). Current single cell RNA-Seq methods still suffer from technical limitations arising from a low yield in RNA to cDNA conversion and bias during cDNA amplification. A single cell typically contains about 1 pg of RNAs, about 10% of which are polyadenylated mRNAs (Tang, et al., 2011). The small starting material is the predominant limiting factor affecting the sensitivity and reproducibility of single cell RNA-Seq (Brennecke, et al., 2013, Ramskold, et al., 2012). Weakly expressed genes often experience ‘drop out’ events or variable expression between samples, which complicates the separation between technical and biological noises. To combat this, external spike-in RNAs can be included at known concentrations before reverse transcription so that technical noise can be estimated using statistical modeling (Brennecke, et al., 2013, Jiang, et al., 2011). They are also frequently used in between-sample normalization tools designed for single cell RNA-Seq (Bacher and Kendzierski, 2016, Ding, et al., 2015, Risso, et al., 2014, Vallejos, et al., 2015). However, some limitations and caveats exist for spike-in RNAs and are discussed elsewhere (Bacher and Kendzierski, 2016, Kolodziejczyk, et al., 2015, Ofengeim, et al., 2017). While IVT is thought to provide more faithful cDNA amplification, PCR is still widely used due to its ease and versatility. However, primer dimers, differences in transcript amplification efficiencies and the exponential nature of PCR can incur substantial technical noises. To improve the quantitative aspect of single cell RNA-Seq, we also see a surge in the incorporation of unique molecular identifiers (UMIs) to reverse transcription primers so that individual transcript can be labeled (Islam, et al., 2014, Kivioja, et al., 2012, Macosko, et al., 2015). The number of unique UMIs aligned to each gene locus will more accurately reflect the mRNA abundance than reads per se, which is susceptible to amplification artifacts. Indeed, methods incorporating UMIs reduced amplification noise and substantially enhanced the accuracy of mRNA quantification, according to a side-by-side comparison of major single cell RNA-Seq methods (Ziegenhain, et al., 2017). The use of nano- or pico-liter

reactions in the microfluidic platforms have also been shown to increase capture rate and accuracy (Islam, et al., 2014, Wu, et al., 2014).

Single cell RNA-Seq analysis of oxygen-sensing cells

Carotid body glomus cells

Until recently, transcriptome profiling aimed at understanding CB glomus cells was performed using the entire CB tissue (Balbir, et al., 2007, Chang, et al., 2015, Fagerlund, et al., 2010, Ganfornina, et al., 2005, Mkrtchian, et al., 2012). This could be problematic, or inaccurate at least. Oxygen-sensitive glomus cell is not the only cell type present within this heterogeneous tissue. In addition, the extremely small CB tissue is highly vascularized and densely innervated by nerve fibers, making it exceedingly difficult to perform a ‘clean’ dissection, especially in smaller animals like mice. The results from probing a whole CB tissue will inevitably be compromised by the dilution of glomus cells as well as the introduction of non-glomus cells. Hence, it is worth obtaining gene expression profile of CB glomus cells at the single cell resolution.

In this section, we will discuss our previous work on isolating and sequencing single mouse CB glomus cells, which generated the first transcriptome profile of these oxygen-sensing cells (Zhou, et al., 2016). We dissociated whole CB tissues into a single cell suspension and picked individual cells (see Zhou et al., 2016 for detailed methods). RNAs were quickly reverse transcribed using oligo(dT) primers, and the resulting cDNAs were poly(A) tailed and further amplified by PCR. To discern cDNAs derived from glomus cells, cDNAs from each cell were used as templates for diagnostic marker genes PCR, and those capable of amplifying known glomus cell markers were selected for sequencing. We identified eight such candidate CB glomus cells from over 200 dissociated CB cells, from which we further constructed Illumina-based next-generation sequencing libraries, sequenced and analyzed. As expected, gene expression profiles of these cells demonstrated some degree of cell-to-cell variation yet they grouped closely together under principal component analysis (PCA), clearly separated from other types of chemosensory cells (olfactory sensory neurons and vomeronasal sensory neurons). The average expression profile of these single glomus cells also showed positive correlations with previous whole CB tissue RNA-Seq and microarray data (Balbir, et al., 2007, Chang, et al., 2015), but demonstrated far greater enrichment of glomus cell-specific transcripts. In other words, while genes abundantly expressed in the single cell data were also expressed in the whole CB data, many of the genes enriched in the whole CB samples may not be highly expressed in the glomus cells (Table 2). As a result, single cell RNA-Seq highlighted genes whose expression in CB glomus cells that were previously underappreciated, many of which were validated by in situ hybridization and immunohistochemistry on adult CB sections. Some of the genes highly overrepresented in CB glomus cells, such as *Olfr78*, *Ndufa4l2*, *Cox4i2*, serve as useful glomus cell markers and may indicate specialized function. Based on the transcriptome data, two prominent features of CB glomus cells immediately came to our attention: G protein-coupled receptor (GPCR) signaling and atypical mitochondrial electron transport chain (ETC). Overall, single cell RNA-Seq of CB glomus cells provided novel information at the molecular level (Fig. 2,

left). This resource will help delineate the oxygen-sensing machinery when combining physiology experiments in genetic mutant animals.

GPCR signaling is used by many sensory cells to transform external information into intracellular responses, such as olfactory sensory neuron, photoreceptor cells and some gustatory cells (Buck and Axel, 1991, Chandrashekar, et al., 2000, Palczewski, 2006). It was not surprising when evidence started emerging in the 1980s suggesting that GPCR signaling, especially the cAMP signaling pathway, is involved in the CB chemosensory process. Multiple studies reported that cAMP increased and potentiated several steps of CB response during hypoxia challenge (Delpiano and Acker, 1991, Lopez-Lopez, et al., 1993, Perezgarcia, et al., 1990, Rocher, et al., 2009, Wang, et al., 1989, Wang, et al., 1991). However, it was not unequivocally supported, and the results varied depending on the assay and species used (Hatton and Peers, 1996a, Hatton and Peers, 1996b, Mir, et al., 1983, Nunes, et al., 2010). Interest in GPCR signaling appears to have faded following the discovery of oxygen-sensitive potassium channels supporting the membrane theory. Hence till now it is still unclear the specific roles of GPCR signaling in CB glomus cells, whether the increase in GPCR signaling is a direct outcome of hypoxia or a result of autocrine or paracrine effects caused by released neurotransmitters and/or hormones. When examining the single glomus cell RNA-Seq data, there was a significant presence of GPCR signaling pathway transcripts, especially those pertaining to the cAMP-mediated signal transduction pathway. *Gnas* (*Gαs*), an adenylyl cyclase activator, was consistently the most abundant transcript in all the glomus cells we had sequenced. In line with this, we also identified many GPCR signaling family members expressing at high levels, including various G proteins, GPCRs, and downstream signaling components and regulators. These data suggest prominent roles of GPCR signaling in CB chemotransduction. Surprisingly, the most abundant GPCR transcripts belonged to the olfactory receptor *Olfr78*. *Olfr78* was also the most differentially expressed gene in CB glomus cells according to the differential expression analysis comparing CB glomus cells with 17 other mouse tissues or cells. *Olfr78* couples to *Gαs/olf* and can be ectopically expressed outside of the olfactory system, such as in the renal juxtaglomerular apparatus to regulate blood pressure (Pluznick, et al., 2013). One question that immediately follows is what roles does *Olfr78* play in CB glomus cells. Does it mediate hypoxia response or facilitate sensitivity to hypoxia, or does it mediate additional chemosensation? To begin answering these questions, we and several groups performed a heterologous screening to search for ligands for this receptor (Aisenberg, et al., 2016, Chang, et al., 2015, Pluznick, et al., 2013, Zhou, et al., 2016). This receptor is highly activated by some short chain fatty acids (SCFAs) such as acetate and propionate. SCFAs are normal constituents of the blood, deriving from fermentation of complex polysaccharides in the gut (Tremaroli and Backhed, 2012). It is possible that SCFAs entering the bloodstream can influence the sensitivity of CB hypoxia response. Chang et al. has also identified *Olfr78* as a highly specific CB glomus cell marker, suggesting that *Olfr78* senses lactate accumulation during hypoxia and indirectly mediates the glomus cells' hypoxia response (Chang, et al., 2015). This is primarily based on their observations that *Olfr78* mutants did not increase breathing frequency during 10% O₂ hypoxia challenge and showed diminished ex vivo CB responses to hypoxia and lactate. Many more questions await further investigation: how does intracellular lactate export and accumulate to a sufficient level at the

cell membrane to activate Olfr78, especially for those dissociated glomus cells under constant perfusion? Also, much is still unknown between Olfr78 activation and glomus cell depolarization. Olfr78 activation can induce cAMP, but apparent lack of cAMP-gated ion channels in CB glomus cells based on single cell RNA-Seq calls for further investigation to elucidate signaling cascade that eventually leads to cellular excitation events. Research directed to this area is much needed to provide clarification on existing data.

Examining the genes significantly overrepresented in CB glomus cells, two atypical mitochondrial ETC subunits (Ndufa4l2 and Cox4i2) encoded by the nuclear genome were highly ranked on this list. Ndufa4l2 and Cox4i2 are induced under long-term and severe hypoxia (Fukuda, et al., 2007, Tello, et al., 2011). However, in both neonatal and adult CB glomus cells, high levels of these atypical mitochondrial subunits were detected, suggestive of constitutive expression (Zhou, et al., 2016). This was an intriguing discovery as it provided the first piece of genetic evidence that CB glomus cell mitochondria, specifically their ETC, are unique at the molecular level. Mitochondria theory is one of the earliest and most classic example of oxygen-sensing theories in CB. The hypothesis stemmed from the facts that mitochondria are the main consumer of oxygen and many mitochondrial ETC inhibitors and uncouplers can mimic the effects of hypoxia on CB (Donnelly and Carroll, 2005, Mulligan and Lahiri, 1982, Mulligan, et al., 1981, Ortega-Saenz, et al., 2003). However, this theory also drew criticisms in several aspects: mitochondria are ubiquitous and have a high affinity for oxygen. These contradict with the facts that CB is a specialized oxygen sensor and can easily detect small drops in oxygen tension. To explain the unique sensitivity of CB glomus cells, experiments have been performed to demonstrate higher sensitivity to hypoxia in CB mitochondria, possibly containing unusual cytochromes with reduced oxygen affinities (Mills and Jobsis, 1970, Mills and Jobsis, 1972, Streller, et al., 2002). Data supporting this theory suggest the presence of atypical mitochondria in CB glomus cells that permit a concomitant increase in NADH and decrease in mitochondrial membrane potential even under moderate hypoxia (Buckler and Turner, 2013, Duchen and Biscoe, 1992a, Duchen and Biscoe, 1992b). However, it was not until the single cell transcriptome profiling of CB glomus cells that molecular evidence supporting the presence of atypical mitochondria existed. This, along with a recent study reporting the involvement of the mitochondrial subunit Ndufs2 in CB-mediated hypoxic ventilatory response, rekindles the once popular mitochondria theory (Fernandez-Aguera, et al., 2015). Based on experiments using conditional Ndufs2 knockout mice, it was proposed that upon hypoxia stimulation, CB mitochondria signal through NADH and reactive oxygen species (ROS) produced at ETC complex I. How may Ndufa4l2 and Cox4i2 contribute to mitochondrial oxygen sensing? Their constitutive and ubiquitous isoforms (Ndufa4 and Cox4i1) are parts of the mitochondrial ETC complex IV or cytochrome c oxidase, the site of oxygen binding and reduction (Balsa, et al., 2012, Fukuda, et al., 2007, Pitceathly, et al., 2013). Ndufa4l2 and Cox4i2 may be located in ETC complex IV of CB glomus cells to modify mitochondria's affinity for oxygen. Previous studies have shown that Ndufa4l2 and Cox4i2 induced under sustained hypoxia can reprogram the mitochondrial ETC to cause functional changes (Fukuda, et al., 2007, Tello, et al., 2011). Under sustained hypoxia, Cox4i2 optimized oxygen consumption and minimized ROS production, while Ndufa4l2 induction led to decreases in mitochondrial oxygen consumption, mitochondrial membrane potential

and ROS production. Intriguingly, Cox4i2 was recently demonstrated to play an essential role in acute oxygen sensing in pulmonary arterial smooth muscle cells, a group of oxygen-sensing cells responsible for hypoxic pulmonary vasoconstrictions (Sommer, et al., 2017). Cox4i2 responded to acute hypoxia with mitochondrial hyperpolarization and increased ROS production. Analogous to findings from Ndufs2 deficient CB glomus cells, ROS generated under acute hypoxia was shown to mediate inhibition of potassium channels and subsequent cellular depolarization in pulmonary arterial smooth muscle cells. The co-expression of the atypical mitochondrial subunits Ndufa4l2 and Cox4i2 may possibly affect cytochrome c oxidase activity and result in a lowered affinity for oxygen. In turn, additional oxygen decreases could more easily affect mitochondrial ETC electron flow, leading to accumulation of NADH and ROS at complex I that signal to membrane ion channels. Further analysis using Ndufa4l2- and Cox4i2-null CB will be required to resolve whether these atypical mitochondrial subunits provide the missing link between unusual CB mitochondria sensitivity and acute oxygen sensing.

Olfactory sensory neuron type B cells

Nematodes contain specialized oxygen-sensing neurons that use atypical heme-containing guanylate cyclases to monitor exterior oxygen level (Gray, et al., 2004, Vermehren-Schmaedick, et al., 2010, Zimmer, et al., 2009). This sensitive signaling pathway has also been adapted by mice to sense environmental CO₂ level using the olfactory system, given its advantageous location (Sun, et al., 2009). While it is prevalently accepted that vertebrates like mice develop several chemoreceptor sites to monitor interior oxygen fluctuations, no evidence existed to suggest the presence of an environmental oxygen sensor before recent studies involving transcriptome analysis of single olfactory sensory neurons (OSNs). A group of non-canonical OSNs called type B cells, characterized by expression of Trpc2 channel and the absence of conventional OSN signaling component Adcy3, was recently found in the mice main olfactory epithelium (Omura and Mombaerts, 2014, Omura and Mombaerts, 2015). To investigate the potential sensory function of this OSN subpopulation, single fluorescent-labeled cells dissociated from the Trpc2-mCherry mice's olfactory mucosa were isolated. Candidate type B cells were identified through single-cell marker genes PCR, the same strategy used to select candidate CB glomus cells. Transcriptome analysis of single candidate type B cells using tag-based cDNA sequencing method (longSAGE) led to the discovery of a poorly-studied soluble guanylate cyclase Gucy1b2 in these cells. Single cell RNA-Seq of mature OSNs also captured type B cells (Saraiva, et al., 2015). Dissociated cells from whole olfactory mucosa were first FACS sorted based on high expression levels of OMP, a marker for mature OSNs. Enriched mature OSNs were loaded onto a Fluidigm C1 microfluidic chip to capture single cells and generate cDNAs based on the Smart-Seq method. Among the 21 OSNs captured, two OSN type B cells distinguished themselves from the remaining canonical OSNs by abundantly expressing 55 differentially expressed genes, with Gucy1b2 being the most highly ranked (Fig. 2, right). Similar to single CB glomus cell RNA-Seq analysis, differential expression analysis methods designed for bulk RNA-Seq were used. While several workflows designed for single cell RNA-Seq data are available, they have not demonstrated pronounced improvement in performance (Dal Molin, et al., 2017, Jaakkola, et al., 2016). The single cell transcriptome data also provided additional evidence that type B cells are indeed a new subpopulation of OSNs with

an alternative transduction pathway and no obvious chemoreceptors, consistent with previous longSAGE results. It was apparent that type B cells must be responsible for sensing fundamentally different stimuli. Given guanylate cyclase family's history in gas detection, it was logical to test whether type B cells respond to oxygen concentration changes. Using fluorescent-labeled type B cells, it was discovered that type B cells can respond to short bursts of reduced oxygen solutions, with rapid and graded calcium rises that were dependent on Gucy1b2. This oxygen-sensing property is mediated through protein kinase G activation and Trpc2 channels. The dose response curves of type B cells revealed an oxygen sensor that operates at much higher oxygen tensions than those of CB glomus cells, making it more prone to monitoring fluctuations in atmosphere oxygen concentrations. While CB glomus cells activation threshold is around 80 mmHg, the majority of type B cells responses fell between 126 to 153 mmHg. Indeed, the CB-mediated hypoxic ventilatory response was intact in Gucy1b2 mutant mice challenged with 10% O₂, consistent with its extremely weak expression (average RPM <3) in single CB glomus cell RNA-Seq data (Zhou, et al., 2016). Type B cells also showed functional significance in helping mice navigate environmental cues as Gucy1b2 mutant mice failed to avoid low oxygen (16% O₂) chamber in a conditioned place aversion test. Without targeted transcriptome analysis of single cells, it is plausible that this small group of specialized sensors for such a small deviation in environmental oxygen could have been easily neglected in the complex olfactory system.

Future applications of single cell RNA-Seq on oxygen-sensing cells

Although transcriptome data of CB glomus cells is now available, more questions can be answered by conducting additional single cell RNA-Seq experiments on CB cells. CB demonstrates complexity in both function and organization. It is known that CB is a polymodal sensor capable of detecting a variety of stimuli, e.g. O₂, CO₂, pH, temperature, glucose and etc. (Kumar and Bin-Jaliah, 2007, Kumar and Prabhakar, 2012, Lahiri and Forster, 2003, Pardal and Lopez-Barneo, 2002b). Can one attribute all these functions to one group of cells? Even among glomus cells, reports have shown not all of them respond to hypoxia or respond at a varying degree (Bright, et al., 1996, Pardal and Lopez-Barneo, 2002a). Instead of selectively sequencing cells defined by marker genes, one can first functionally categorize CB cells based on response profiles before performing single cell RNA-Seq. Subsequent post hoc transcriptome comparisons can help reveal the molecular basis for such variations. An apt example is Patch-Seq, a protocol developed for generating transcriptome profiles from single neurons after whole-cell patch-clamp recordings (Fuzik, et al., 2016). This strategy is desirable for discovering predictive molecular markers for a specific modality, but it is limited in that functional assays may damage the cell and the quality of the RNAs.

Organization wise, the CB parenchyma contains glomus cells, sustentacular cells, endothelial cells, nerve fibers, fat cells and connective tissues. This is further complicated by recent progress suggesting CB as a site of neurogenesis, where sustentacular cell-derived stem cells can transform to glomus cells under chronic hypoxia (Pardal, et al., 2007, Platero-Luengo, et al., 2014). It would be interesting to perform single cell RNA-Seq on these stem cells or CB cells from chronic hypoxia-treated mice. Understanding the gene-regulatory modules controlling the conversion of oxygen-insensitive sustentacular cells to glomus cells

may offer clues on the identity of glomus cell oxygen sensors. One may also obtain a better understanding of the lineage relationship among these cells by using newly developed workflows that reconstruct lineage trajectories using single cell RNA-Seq data, such as Slingshot, Wishbone, and Monocle (Fletcher, et al., 2017, Setty, et al., 2016, Trapnell, et al., 2014). These algorithms can sort cells into different subtypes, infer their interrelationships and reorder them on a pseudotime trajectory. This is based on the assumption that these cells represent different stages of a dynamic process that reflect through gene expression changes. This approach has proven to be especially informative for studying developing or differentiating tissues (Fletcher, et al., 2017, Hanchate, et al., 2015, Linnarsson, 2015).

Alternatively, one can study the CB by randomly sampling a large group of single cells so that molecularly distinctive subgroups and novel biomarkers can be classified in a data-driven manner without a priori knowledge. Growing evidence reveal great cell-to-cell gene expression heterogeneity exists among what once thought to be a homogeneous group of cells. Is it possible that we are oversimplifying glomus cells, especially when we equate tyrosine hydroxylase positive cells with glomus cells in most of our studies? Now that massively parallel single cell RNA-Seq has become practical, one can attempt to decompose CB. This is an area with a promising outlook. Studies have already been done on tissues as complex as the brain and some sensory tissues to successfully reveal subpopulations with new markers or new functions (Burns, et al., 2015, Cuevas-Diaz Duran, et al., 2017, Furlan, et al., 2016, Gokce, et al., 2016a, Macosko, et al., 2015, Usoskin, et al., 2015, Zeisel, et al., 2015b). Using microdroplet-or microwell-based platforms, one can perform low depth sequencing of hundreds to thousands of CB cells in one experiment. This dramatically increases sample size to compensate for known noisiness related to single cell RNA-Seq itself. Shallow sequencing has demonstrated sufficient power to distinguish known and novel cell types while controlling cost (Gierahn, et al., 2017, Klein, et al., 2015, Macosko, et al., 2015, Pollen, et al., 2014, Zheng, et al., 2017, Ziegenhain, et al., 2017). Sequencing hundreds to thousands of CB cells will generate large datasets that can be unbiasedly analyzed using unsupervised clustering methods. Due to the high-dimensionality of RNA-Seq data, popular dimension reduction methods such as PCA and t-Distributed stochastic neighbor embedding (tSNE) can be used to detect and visualize subpopulations of single CB cells (Furlan, et al., 2016, Klein, et al., 2015, Macosko, et al., 2015, Treutlein, et al., 2014). Both of these methods can identify dimensions that capture the most variation within the data and project them to a lower dimensional space so that one can more easily visualize the inherent structure, but the latter uses a non-linear transformation to better preserve local distances (Amir, et al., 2013, van der Maaten and Hinton, 2008). Aside from dimension reduction, distance-based clustering methods such as hierarchical clustering and k-mean clustering can also be applied to identify CB subpopulations and their associated gene sets. Increasing numbers of computational methods designed specifically for single cell RNA-Seq are being developed, and are reviewed in greater details elsewhere (Bacher and Kendzierski, 2016, Poirion, et al., 2016, Rostom, et al., 2017).

In addition to CB glomus cells, other oxygen-sensing cells can also benefit from single cell sequencing technology. These cells are often in low quantity or located within complex structure. Single cell RNA-Seq can overcome such obstacles and serve as a discovery-based screening for candidate oxygen-sensing molecules. Several small groups of arterial

chemoreceptors called the aortic body are sparsely distributed near the aortic arch. Aortic body also contains structurally similar glomus cells that are capable of reacting to drops in arterial oxygen tension, but it is not certain if they share the same oxygen-sensing mechanism as CB glomus cells (Brophy, et al., 1999, Piskuric and Nurse, 2012). Aside from the arterial circulatory system, the pulmonary system is also a critical component of oxygen provision. The lung has developed specialized pulmonary arterial smooth muscle cells and neuroepithelial bodies capable of responding to acute hypoxia (Cutz, et al., 2013, Wang, et al., 2005). Furthermore, the chromaffin cells in fetal and neonatal adrenal medulla are also sensitive to hypoxia (Rico, et al., 2005, Weir, et al., 2007). Interestingly, a series of studies have indicated similarities between these cells and CB glomus cells, such as oxygen-sensitive potassium channels and sensitivity to mitochondrial inhibitors (LopezBarneo, 1996, Mojet, et al., 1997, Nurse, et al., 2006, Weir, et al., 1994, Youngson, et al., 1993). Oxygen-sensing property has also recently been attributed to astrocytes in the central nervous system (Angelova, et al., 2015). The activation threshold of these cells is at around 40% of that of CB glomus cells, but this lower sensitivity is consistent with the local oxygenation level in the brain. Similar to peripheral oxygen sensors, astrocyte mitochondria were inhibited under hypoxia, resulting in mitochondrial depolarization and ROS generation. Single cell RNA-Seq data for astrocytes have become available thanks to previous efforts in single cell sequencing of brain tissues, some of which have successfully identified astrocyte populations (Dulken, et al., 2017, Gokce, et al., 2016b, Zeisel, et al., 2015a). Comparing transcriptomes from different oxygen-sensing cells may help reveal a common molecular signature for detecting oxygen fluctuations.

Conclusion

Prior to single cell RNA-Seq, researchers only had an incomplete or blurry gene expression profile of a tissue's subpopulation, based on either assessments of selected genes or a vague population average of all possible cell types present. Without comprehensive knowledge of the molecules that encode each cell's functional identity, it was a great challenge to work with rare or heterogeneous samples such as specialized oxygen-sensing cells, brain cells, embryonic cells and tumor cells. Single cell RNA-Seq has enabled 'high-resolution' examination of such tissues and helped unravel key insights regarding their transcriptomes (Cuevas-Diaz Duran, et al., 2017, Muller and Diaz, 2017). In little over eight years, remarkable advancements have been made to increase both the accuracy and throughput of this innovative tool, allowing simultaneous quantifications of hundreds of transcripts in thousands of cells at once. Growing applications have also opened the doors to new questions about previously unappreciated cell type heterogeneity and catalyzed a series of unsupervised analytical tools for processing the overwhelmingly large datasets. By applying single cell RNA-Seq to CB glomus cells, we obtained the first transcriptome profile of these cells, providing a list of genes that may be involved in glomus cell function. We have also unexpectedly discovered an olfactory receptor and two atypical mitochondrial subunits as highly and specifically expressed in CB glomus cells, providing means to test major theories of CB oxygen sensing. Single cell RNA-Seq has also made a major contribution by adding OSN type B cells to the repertoire of acute oxygen-sensing cells in mice. The discoveries of these cells and the underlying oxygen-sensing mechanism were only made possible by

single cell transcriptome profiling. With rapid improvement in both single cell sequencing and bioinformatics technology, we expect to see many more exciting studies in the field of oxygen-sensing cells.

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Figure 1.

Overview of single cell RNA-Seq workflow. **a**, four major single cell isolation methods. From left to right, manual pipetting from a single cell suspension, laser micro-dissection from a tissue section, FACS sorting of fluorescent-labeled cells, microdroplet isolation of a single cell with a capture bead containing cell-specific barcode, unique molecular identifier (UMI), and oligo(dT) primer. **b**, a single cell is lysed to release polyadenylated mRNAs (purple) for reverse transcription, followed by PCR- or IVT-based cDNAs amplification and preparation of next-generation sequencing library. For PCR-based approaches, reverse transcription involves poly(A) tailing or template-switching. Once the first-strand cDNA is generated, a poly(A) tail is added to the 3' end so that small quantity of cDNAs can be further amplified using oligo(dT) primers. Template-switching methods use reverse transcriptase that adds several C nucleotides only when the enzyme reaches the 5' end of the mRNA transcripts. Primers containing complementary G nucleotides will then anneal and serve as an extended template, allowing selective amplification of these transcripts. For IVT-based approaches, a T7 promoter sequence is added to the oligo(dT) primer during reverse transcription to enable subsequent recognition by the DNA-dependent RNA polymerase.

Figure 2.

Illustrations of single cell RNA-Seq of oxygen-sensing cells. Oxygen decrease activates CB glomus cells (left) and OSN type B cells (right). **Left**, lacZ staining of a carotid artery bifurcation with blue precipitation marking the Olfr78-positive CB glomus cells. The heterogeneous CB structure is depicted: oxygen-sensing glomus cells (blue) glomeruli are enveloped by sustentacular cells (green) and nerve fibers (grey); blood vessels (red) penetrate through the CB parenchyma to ensure oxygen supply; the CB parenchyma is also surrounded by fat cells (yellow) and other connective tissues (not shown). Single cell RNA-Seq study of CB glomus cells identified the expression of the Olfr78 receptor, stimulatory G protein G α s, and two atypical mitochondrial ETC subunits (Ndufa4l2, Cox4i2). The presence of these proteins may influence cAMP, ROS and NADH level during acute hypoxia. **Right**, the olfactory epithelium is a heterogeneous tissue containing different cell types: a new OSN subtype called type B cell (red) was identified based on single cell RNA-Seq; Type B cells can respond to environmental oxygen decrease while other canonical OSNs (blue and yellow) respond to volatile odors; basal cells (green) and supporting cells (light orange) also contribute to the complexity of the olfactory epithelium. Single cell RNA-Seq discovered that soluble guanylate cyclase Gucy1b2 is highly specific to type B cells. Gucy1b2 and ion channel Trpc2 are critical mediators of Type B cell oxygen-sensing, possibly involving increase in cGMP during hypoxia.

Table 1

A comparison between bulk RNA-Seq and single cell RNA-Seq

	Bulk RNA-Seq	Single cell RNA-Seq
Dissociation	No	Yes for tissues
Special equipment	No	Yes for most cell isolation methods
RNA input	High	Low
RNA species	Various	Only polyadenylated RNAs
Workload	Light	Laborious, but improved using recent cell isolation and barcoding technology
Fidelity	High	Low for weakly expressed genes
Detection sensitivity	High	Low for weakly expressed genes
Technical noise	Low	High for weakly expressed genes
Biological noise	Low	High
Resolution	Population level	Single cell level, can recreate bulk expression by pooling 10 or more cells
Bioinformatic analysis	Easy, many established algorithms and statistical methods	Complicated, some workflows tailored to single cell RNA-Seq data
Application	Average gene expression comparison between bulk tissues	Rare cell types, heterogeneous samples, random monoallelic expression, lineage development and other dynamic process

Table 2

Top 1% genes and their corresponding ranking averages in different transcriptome studies

	Average ranking	
	C57BL/6J whole CB RNA-Seq	DBA/2J whole CB microarray
Top 1% genes from single glomus cell RNA-Seq	top 5%	top 10%
	Average ranking in single glomus cell RNA-Seq	
Top 1% genes from C57BL/6J whole CB RNA-Seq	top 28%	
Top 1% genes from DBA/2J whole CB microarray	top 46%	