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Review

A compendium of single cell analysis in aging and disease

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Abstract: Cell is the fundamental structural and functional unit of complex multicellular organisms. Conventional methods which involve average analysis of cells in bulk populations can undermine physiologically significant cell populations, whereas analysis of cells at a single cell level may reveal unique biomarkers and other mechanisms that govern the genotype and phenotype in various physiological processes in presumed homogenous cell populations. Cellular abnormalities such as irregularities in cellular mechanisms have been linked to human aging and other major diseases including neurodegenerative, vascular, autoimmune, and cancer. Aging is a functional decline associated with various diseases in an organism, majorly arising from cellular abnormalities. Single cell analysis (SCA) which involves isolation and study of single cell proteomics, genomics, transcriptomics and metabolomics which enables research of cellular abnormalities with a molecular resolution, is gaining recognition in the research of human aging and disease. The advances in SCA are producing breakthrough information about cellular heterogeneity, disease progression, cellular microenvironment and its interactions, early diagnostics, improving precision medicine through high throughput drug screening and discovery of novel biomarkers; combinedly, these advances exhibit the potential of SCA to study of human aging and disease. Primarily, we review the role of SCA in understanding cellular mechanisms involved in aging and other major diseases including neurological, vascular, autoimmunity and cancer. Secondly, we also include review of SCA role in studying cell adhesion mechanisms which are involved in tissue development and maintenance and disease progression. Finally, SCA potential to empower precision medicine and its overall challenges along with future directions are discussed.

Keywords: single cell analysis; aging; single cell RNA-seq; disease; adhesion; cancer

1. Introduction

All Multicellular Organisms are built on basic units called “cell”, a fundamental unit of structure, function, and organization. Understanding cells and interactions with its microenvironment can help gain insights into the development and progression of a disease, the effects of human aging, and cellular differentiation. SCA is the study of individual cells isolated from various tissues and analyzed for their “Omics” profiles including genomics, transcriptomics, proteomics and metabolomics. The conventional techniques such as western blot, mass spectrometry and sequence profiling of cells for detection of biomarkers, drug treatment outcomes and other pathologically significant molecule investigation, use the average readouts of bulk cell populations which mask the underlying heterogeneity and microenvironments of the cells. Unlike conventional techniques, SCA techniques including Atomic force microscopy, Microfluidic studies, Single cell RNA-sequence (ScRNA-seq), etc., can investigate the events occurring at a single cell level and improve recording events of individual gene expression profiles, cellular heterogeneities and other cellular activities previously unknown [1]. The small size of the cells and low amounts of analytes from cellular processes still pose great challenges in recording those events, presenting the scope for development in SCA techniques.

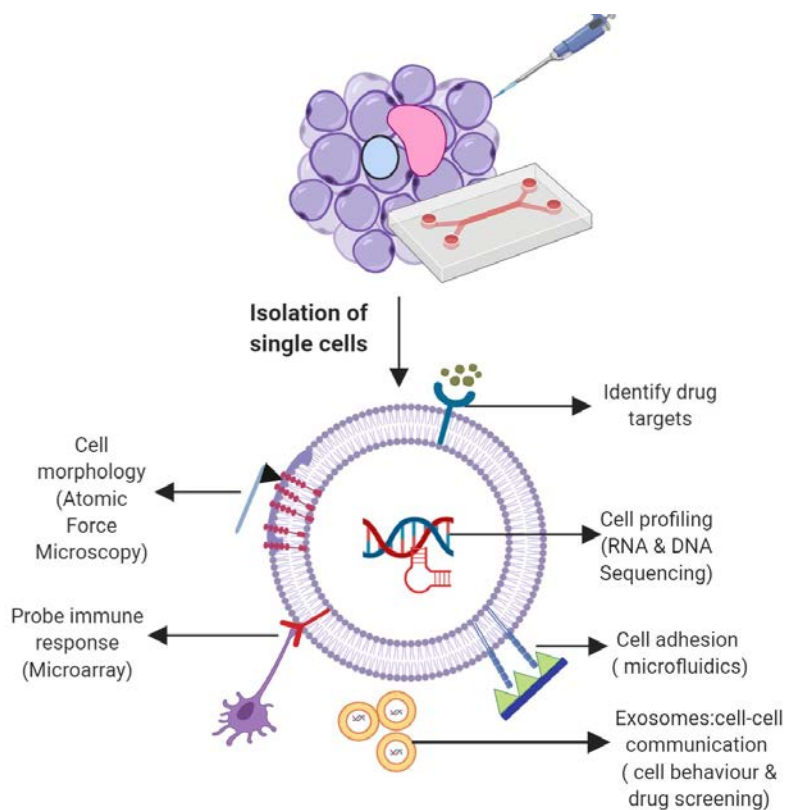


Figure 1. Single cell analysis overview.

Cell undergoes complex processes of migration, mitosis, exocytosis and endocytosis of various molecules throughout its lifespan and, during these processes there are many events occurring in the cellular environment to maintain homeostasis. Research indicates that primitive life of the cell was formed in a symbiotic relationship with mitochondria, previously believed to be a separate living entity, was recently found as cell-free entity in blood which may be involved in cell-to-cell communications and other unexplored viral fragments such as retrotransposons fused into human genome; together, these features exhibit the high complexity involved in cellular mechanisms [2–8]. These complex cell systems undergo transitions from one physiological state to another during differentiation and maturation based on their interactions with its micro-environments, commonly referred to as “Cellular plasticity”. SCA has shown to be a valuable tool in characterizing the cellular plasticity. For instance, Mitra Mjtahedi et al. have shown a theory based approach to finding the tipping point of when a multipotent progenitor cell will differentiate into myeloid and erythroid cells using single cell profiling [9]. Also, SCA has shown a rather unique cell-division independent heterogeneity in phenotypic hematopoietic stem cells, revealing a new perspective on differentiation without cytokinesis; which could be further modeled as a parameter to study any such occurrences in other pathologically significant cell populations such as cancer cells [10]. Similarly, SCA techniques such as Drop-Seq, *in situ* barcoding, Lab-on-a-chip, single-cell RNA sequencing techniques like Smart-Seq2, laser capture microdissection, etc., have the potential to study complex functions in cellular populations with single-cell resolution to uncover novel cellular features including cell phenotypes, biomarkers, differentiation profiles and therapeutically significant sub populations, which are specially advantageous over conventional cell profiling techniques by excluding contaminating cells and considering rare cell populations [11–13]. Cellular plasticity aids in organ formation during embryogenesis, epithelial-mesenchymal transition (EMT), intermediate hybrid states, etc., where cell must make “fate decisions” majorly depending on its environmental factors including pH, redox elements, antigen presentation cytokine gradients, epigenetic modifications, etc., and dynamics between those factors and fate decisions are still largely unknown [14–16]. Fate decisions including upregulation of surface marker expressions, phenotype changes, epigenetic modifications, adhesion, etc., are linked with many dynamic processes in embryo development, cell division and differentiation, cell migration, intercellular communication via exosomes and other physiological activities. When these dynamic processes are out of a control or enter a deregulated state, they trigger the onset of aging and disease progression. SCA techniques have emerged to be an important tool in illuminating these processes occurring at a single cell level and help us understand these irregularities in cellular mechanisms [1]. Ping Hu et al. have reviewed most of the single cell isolation techniques [17] and Lucas Armbrrecht et al. have detailed various contemporary single cell analysis tools and techniques [18]. This review will be a compendium of SCA with a focus on its role in understanding aging and other major diseases including neurological, vascular, autoimmune and cancer. A brief workflow of SCA from isolation of single cells, its analysis and potential outcomes is given in Table 1.

Table 1. Workflow of Single cell analysis.

1. Isolation of single cells	2. Single Cell Analysis	3. Outcome
<ul style="list-style-type: none"> • Pipetting [19] • Fluorescence, magnetic activated cell sorting (FACS & MACS) [20] • Laser capture microdissection (LCM) [21] • Microfluidics isolation [22] • Surface based cell adhesion [23,24] • Raman optical tweezers (ROT) [25] • SORT-seq [26] • Microhip platform for single cell exosomal vesical profiling [27] 	<ul style="list-style-type: none"> • Microfluidic based wells, traps and patterns [24,28] • Optical methods: live cell imaging (SCOPE-Seq [29]), fluorescence techniques [30] • ELISAs • Acoustofluidic sensing [31–33] • Electrochemical analysis [34–36] • Mass Spectrometry [37–41] PCR based methods [42] • Atomic force microscopy [43,44] • Nanomotors [45,46] • Raman spectroscopy [47,48] • Fluorescent in situ Hybridization (FISH) [49–51] • PNA-FISH [52] • Single cell RNA sequencing (ScRNA-seq) • Div-seq [53] 	<ul style="list-style-type: none"> • Probe Cellular heterogeneity [54,55] and Cellular functionality [56] • Track Cell lineage [57,58] • Cell-substrate response [59–61] • Cellular level drug response [62,63] • Invaluable tool in human atlas project. • Aid in Anti-Microbial research [60,64] • Identify novel drug targets [65]

2. SCA in aging studies

Cells are functionally and structurally regulated to perform specific tasks and maintain functionality in tissues, e.g., hematopoietic stem cells (HSC) ability of differentiation into various blood cells and immune cells to maintain homeostasis among organs, neural stem cells (NSC) in generating new neurons for various brain functions including learning, memory, etc. Similarly, cells of other body systems have their own functions which are tightly regulated to maintain tissue homeostasis and cell behavior changes with age reflecting on the structure and functionality of a multi-cellular organism, typically ensuing detrimental effects. Cells survive constantly by repressing apoptosis—a physiological form of cell apoptosis via intercellular signaling and self-destruct when they are damaged and unusual to the system, by triggering apoptosis [66]. When the regulatory mechanisms governing the innate functioning of cells are compromised and beyond repair due to various factors including oxidative stress and mitochondrial dysfunction, the onset of senescence triggers, which comprises of irreversible arrest of cell cycle avoiding division and apoptosis, acquiring DNA damage such as telomere shortening, epigenetic modifications and morphological changes. These physiological changes in cell impart chronic inflammation and disrupt tissue homeostasis leading to reduced organ functionality and disease, mostly seen in *human aging*. Isolation and study of senescent cells and damaged progenitor cells like HSC and NSC to identify significant biomarkers with molecular resolution is critical to address various

pathophysiological health issues which develop with age. Telomere which protects the ends of chromosome is shortened after numerous cell division cycles reducing cell's proliferative abilities and eventually acquire senescence, telomerase reverse component (TERT) which is critical in telomerase repair, has very low expression inside a cell, making it difficult to study with conventional bulk cell analysis. Ravindranathan et al. have developed single-molecule techniques in combination of RNA scope coupled PNA FISH detection of TERT based addition of telomere units onto chromosomes in single cells; which creates a platform to study effects of age related drugs on senescent cells [52].

SCA techniques like single-cell RNA-seq have been crucial in identifying varied functional states of single cells in presumed homogenous functional state in bulk cell populations. HSC, especially long-term reconstituting HSC (LT-HSC) which are the most primitive pool of stem cells with potential to differentiate into various blood cell types and also an important indicator of regenerative potential is normally seen to decline with age. SMART-seq study of comparative analysis of LT-HSCs single cells of young and old mice probed for their cellular state changes found old LT-HSCs to spend less time in the G1 phase cell cycle when compared to young HSCs along with reduced cell cycle-related component expression in old HSC contrary to the expression observed at bulk population level, which is correlated to lower rate of differentiation and higher accumulation of old LT-HSC with possible detrimental effects on tissue homeostasis [67]. Morita et al. used single-cell culture and transplantation assay to study hematopoietic stem cells (HSC) differentiation potential in primary and secondary recipient mice (HSC transplanted from primary mice); where a higher number of HSC with CD150 marker associated with differentiation potential of HSC into erythroblast/megakaryocyte were seen in secondary recipients with a shift towards lymphoid nature than in primary recipients, showing an underlying hierarchy and complexity in reconstitution capability of HSC [55]. Similar to blood cells generated from HSC, adult neurons are generated from NSC to maintain tissue homeostasis in various brain regions helping in learning, memory and repair [68]. Single-cell RNA-seq of NSC cells isolated using Fluorescence activated cell sorting (FACS) and Immunostaining, studied in young and old mice revealed the increased infiltration and clonal expansion of CD8⁺ CD4⁻ T cells in old mice as well as human NSC niche than the younger ones and interferons secreted by those infiltrated T cells showed inhibitory effects on NSC subtypes in old mice revealing possible clues related to age-associated cognitive impairment due to impaired NSC populations [69]. Also, recent single cell transcriptome profiling using single cell RNA-seq technique study with a focus on ribosome biogenesis pathway in different neuronal cell types argue that aging process may be more varied and gradual than an identical molecular program shared across cell types, which shows the advantages of SCA in probing cellular landscape versus the conventional analysis in bulk population of cells [70]. SCA studies which used miRNA Microarray Affymetrix chips in analyzing miRNA species from hypothalamic Neuronal Stem cells (htNSC) in cerebrospinal fluid of mouse models showed age-associated reduction in htNSCs, and the introduction of genetically engineered hypothalamic stem/progenitor cells which survive in an inflammatory environment of aging have achieved noteworthy aging retardation and lifespan extension in mice, suggesting that htNSC cells in part control brain aging speed through exosomal miRNAs [71]. Lab on a Chip device such as neuro-optical microfluidic platform reported by Kim YT et al. demonstrate the ability to isolate and analyze neuronal activities including degeneration, regrowth and functional activities which can help understand the changing physiological activity and detrimental effects of aging at a single-cell level [72].

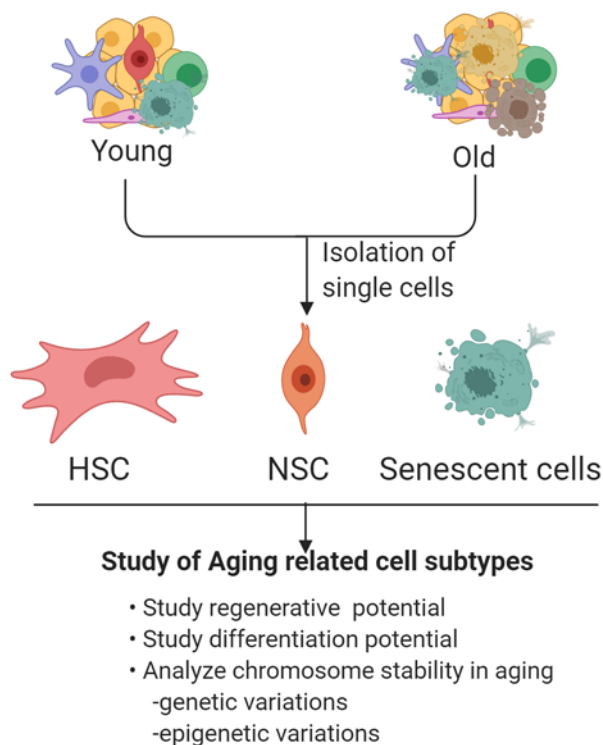


Figure 2. SCA role in aging studies.

Somatic genomic variations referred to as chromosomal instability have shown to play a key role in effecting human neuronal diversity and brain related diseases [73] with intercellular epigenomic variations such as in histone acetylation and DNA methylation which alter and mediate gene expressions in neurons of central nervous system leading to various effects on aging brain and development of neurodegenerative disorders [74,75]. SCA methods like Fluorescent *in situ* hybridization (FISH), immune-FISH, Spectral Karyotyping (SKY), genome microarrays, and other single cell genome and molecular staining techniques can help uncover somatic variations and its effects linked to brain aging [75,76]. A combination of SCA techniques; single nucleus RNA-Seq (Smart-Seq) and Div-Seq are being employed to identify and profile newborn neurons in the adult brain and spinal cord while also track their transcriptional dynamics [53,54]. These latest SCA techniques can give a boost to the quest in finding molecular mechanisms [77], major pathways [70], morphological changes and epigenetic modifications [78] that contribute to the aging processes in brain. Studying the characteristics of these regenerative and their related senescent cell populations with single cell resolution can help model drug targets to understand aging process.

3. SCA in study of disease

Major studies to understand pathological diseases at cellular level includes examination of proteostasis, intracellular signaling through proteins, RNA metabolism changes, energy metabolism, the influence of microenvironments on stem cells and diseased cells, variations in stem cell differentiation,

cellular cytokine profiles, etc. [79], which propels development of molecular therapeutics. Due to the complexity of the disease etiology, such as the genetics and environmental factors which influence various downstream molecular mechanisms across the cell, multi omics study such as SCA can be highly beneficial to provide meaningful insight into the disease and to tailor precision medicine. Hasin et al. have discussed in detail on design considerations of multi omics studies and its data integration of various techniques to investigate information flow in a disease [80]. Along with single cell sequencing techniques, new-generation mass spectrometry techniques such as Fourier transform ion cyclotron resonance (FTICR) [37,38], time-of-flight mass analyzers [39,40], nano-flow electrospray ionization (nanoESI), etc. [41,81] provide insights in understanding functionally important proteins, peptides and metabolites generated from differential gene expressions of pathologically significant cell populations. Role of SCA in neurodegenerative diseases, vascular, autoimmune, and cancer will be discussed in this section.

3.1. SCA role in neurodegenerative diseases

Neurological diseases and neurological disorders are linked to abnormalities of cells associated with brain. Various global initiatives like Human Brain Project, China Brain Project, BRAIN initiative, Japan's Brain Mapping by integrated Neurotechnology's for disease studies, etc. are employing SCA techniques to understand the genotype and phenotype of various known and seemingly unknown cell types. Single-nucleus RNA sequencing methods with iterative clustering and classification of single-neuron data are deployed in categorizing and comparing single nuclear transcriptomes to reveal unknown neuronal cell subtypes in human brain [82]. DNA analysis of post-mortem brain samples of schizophrenia patients with multicolor FISH technique detected chromosomal aneuploidy and trisomy compared to diploid in normal patients revealing neurological disorder markers in cells which are hard to acquire from whole tissue samples [50]. A hypothetical model of GIN'n'CIN (genomic instability and chromosome instability) suggests neural aneuploidy produced in early brain development can lead to various genomic variation related diseases including Alzheimer's disease [76]. Gene expression analysis revealed pathogenic alterations in common transcripts such as glutamatergic neurotransmission, synaptic-related markers, protein phosphatases and kinases and neurotrophin receptors, in vulnerable brain cell types of Alzheimer's diseases [83]. Single cell profiling of dopaminergic neurons has identified genetic modifiers in Parkinson's disease patients in a genome-wide expression studies combined with association analysis of different patient samples showing the potential of SCA to assess genetic risk factors in neurodegenerative disorders [84]. Human brain transcriptome diversity study on fetal and adult neurons of human cortical neurons has discovered a subset of adult neurons which express major histocompatibility complex class I genes which makes them exposed to various immune responses unlike fetal neurons [85]. James Eberwine et al. report of a system where they have successfully cultured brain cells with its original robustness and performed single cell transcriptomics on 300 individual single cells to study their single cell transcriptomics and were able to identify brain cell types of oligodendrocytes, microglia, neurons, endothelial cells and astrocytes based on cell marker expressions and morphology [54]. Systems like these can be used to understand characteristics of cell types in normal and diseased brains, which can help illuminate the plasticity and underlying pathologically significant biomarkers. Microfluidic compartmentalization technology developed by Ayaka Yamada et al. included construction of controlled 3D microenvironments to culture brain cell

types like neurons and glial cells which can be analyzed for drug responses and other pathophysiological activities in natural environments unlike conventional *in vitro* cultures which does not truly mimic 3D *in vivo* microenvironment [86].

3.2. SCA role in vascular diseases

Vascular related disease is very high in age-related deaths where vascular wound healing impairment due to lack of neovascularization and atherosclerosis plaque formations in vasculature presumably caused by inflammation are quite prominent. SCA of progenitor cells such as HSC, endothelial progenitor cells involved in regeneration, repair and other inflammatory microenvironment can help improve the understanding in vascular diseases and reveal novel molecular markers which can be targeted for therapeutic purposes. Single-cell expression profiling in atherosclerotic and inflammatory significant vascular cells of smooth muscle cell (SMC) and endothelial cell subpopulations has revealed heterogeneity in G-protein-coupled receptor (GPCR) expression patterns in specific cell subtypes compared to conventional expressional systems where RNA/cDNA is pooled from bulk populations disabling identification of novel subpopulations. SCA techniques like mass cytometry (CyTOF) in combination with clustering algorithms have detected various immune cell populations and low frequencies of atheroprotective B1 cell subsets with no spectral overlap unlike FACS in contrast with dominant B-cell content detected by flow cytometry from whole atherosclerotic aortas of mice [87,88]. LCM techniques can be employed to dissect desired phenotype of cells, e.g., macrophage rich areas of atherosclerotic plaques, which can be further analyzed by ScRNA-Seq in identifying new immune cell subsets and their functions in atherosclerotic progression. Finding various molecular pathways and other therapeutic targets associated with ischemic heart diseases is highly sought after as the disease involves remodeling of the damaged cardiac tissue which lead to complex cardiovascular conditions. SORT-seq technique employed by Gladka et al. enable study of single-cell gene expression data of both healthy and ischemic adult mice hearts. Although, bulk RNA sequencing showed upregulation of Ckap4 gene in ischemic heart, single-cell sequencing using SORT-seq revealed that Ckap4 upregulation is specifically seen in activated fibroblasts of ischemic hearts, which shows the advantages of SCA in identifying novel pathological markers for diagnosis [26,89]. Also, SCA techniques including scRNA-seq and Single Cell Assay for transposase-accessible chromatin using sequencing (scATAC-seq) are employed to understand cardiac regeneration by studying different regulatory networks and fate decisions in stem cell differentiation of embryonic heart development in mice [90].

3.3. SCA in Auto Immune diseases

Both innate and adaptive immune systems play a major role in our body's defense against harmful pathogens and their associated diseases. Immune dysfunction such as acting on self-antigens in these systems leads to autoimmunity and related autoimmune diseases including diabetes, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), etc., which have been studied extensively to understand underlying immune cell mechanisms. Different immune cell types including regulatory CD4⁺ T cells, Dendritic cells, Cytotoxic CD8⁺ T cells are involved in the homeostasis and immune tolerance mechanism. Major auto immune diseases have immunomodulatory and immunosuppressive agents as treatment regimen which has varied results from patient to patient because of the complexity in the

immune cell landscape. SCA technology is gaining significance in the study of these cell types for their heterogeneity, predictive biomarkers and multi-omics in the context of auto-immune diseases. Alterations in dendritic cells (DCs), e.g. absence of DCs in animal models has shown to induce autoimmunity and other associated development of autoimmune diseases [91]. Distinctive subsets of DCs involved in the immune response of DCs to treatments in patients may be associated with varied response to treatments. Ashton et al. studied DCs gene expression in healthy, RA and SLE by single-cell sorting using BD FACS and then analyzed the gene expression using single-cell RT-PCR. They found increased levels of gene expression of CD86 in CD1c+ cDCs, TLR7 and IRF7 in pDCs and TAP1 and IFNAR1 in RA and SLE DC subtypes [91]. These results correlate with previous findings of increased capacity to activate T cells, produce type I IFNs and processing of antigens respectively; combinedly showing that single cell analysis of Immune cell subtype can reveal unique biomarkers which can be used to design treatment regimes.

Loss or losing functionality of insulin-producing beta cells in the pancreas due to inflammatory infiltrates results in deregulation of blood glucose levels leading to a disease condition called *diabetes mellitus* and eventually other pathological conditions in humans. Evaluating the heterogeneity in the beta cells and other pancreatic cells can reveal the plasticity in the pancreas towards understanding the trigger for diabetes and its progression. With the use of single-cell analysis technologies including single-cell transcriptomics and single-cell RNA-seq, Tritschler et al. have discovered a proliferative β -cell subpopulations in pancreatic cells which can be targeted for enhancing proliferation and maturation, similarly SCA can probe for any potential cell subpopulations with regenerative capacity to form new beta cells, in the diseased state [3]. Single cell transcriptome analysis has revealed heterogeneity of progenitor marker expressing acinar cell subpopulation of acinar cells in the pancreas which shows the regeneration potential in acinar cells [92]. Findings like these can broaden the information on developmental processes in pancreatic cell niche and help us characterize cells and develop regenerative cell therapies for treating diabetes. In search for new biomarkers for early detection of Type 1 diabetes (T1D) Kallionpaa et al. studied RNA-seq of Autoantibody⁺ prediabetic children CD4⁺, CD8⁺ and B-cells which are linked to T1D pathogenesis and in their study they report upregulation of CD52 in only CD4⁺, downregulation of IFNG CD8⁺ cells and upregulation of pro-inflammatory Il-32 secretion in β -cells; which shows the inflammatory niche involved in T1D and potential biomarkers [93]. Studies like single cell profiling of target cell types have the capability to reveal biomarkers for early detection of diabetes.

Various genetical and environmental factors are involved in the development of SLE. Improper balance between apoptotic material disposal and cellular apoptosis leads to chronic inflammation and eventually to an auto immune disease, SLE. As immune response against autoantibodies and anti-double strand DNA antibodies is highly involved in the development of SLE, immune profiling in patients could reveal novel biomarkers for targeting SLE. In that context, Jin Zhongbo et al. studied the individual monocyte cell subtypes from the blood samples of SLE patients for their single-cell gene expression. In contrast to bulk cell analysis which show correlation between disease activity and type I IFN induced gene expression, single cell gene expression analysis in this study showed patterns independent of disease activity and treatment, hinting on the reevaluation of known biomarkers in identify SLE [94]. In another study, William et al. showed the advantages of mass cytometry studies in profiling single-cell phenotypic and functional aspects of SLE associated target cells. They have found a distinct cytokine signature in monocyte phenotype of CD14^{hi} with increased levels of monocyte chemoattractant protein-1 (MCP1), macrophage inflammatory protein-1 β (Mip1 β), and interleukin-1

receptor antagonist (IL-1RA) [95]. Overall, with emerging technologies in single cell analysis such as scRNA-seq, Mass cytometry, microfluidics, etc., new insights into immune cell plasticity such as recently discovered innate lymphoid cells (ILC) which are equipped to sense environmental threats [96], can be probed and aid discovery of cellular mechanisms involved in autoimmune diseases.

3.4. SCA role in cancer

Cancer is a disease that arises due to deregulated cell growth and leads to the formation of tumors which interfere with the organ functionality. Cell heterogeneity has been identified within the same tumor revealing the complex traits of cancer cells and isolation and study of these heterogeneity in cancer cells is critical to develop targeted medicine [97,98]. Molecular profiling of therapeutic targets such as lymphotoxin activation gene 3 (LAG3) and PD-1 which binds to MHC II molecules with high affinity and is highly upregulated in exhausted T cells and other tumor infiltrating lymphocytes, serving as an immune checkpoint blockade and blocking of LAG3 has shown to improve anti-tumor T cell responses. SCA technologies such as single-nucleus sequencing and Fluidigm C1 single chip have demonstrated the ability to automate single-cell lyses, RNA extraction, and cDNA synthesis in parallel. SCA techniques like these can be employed to process and detect complex EMT/MET molecular markers/signals and other checkpoint proteins such as LAG3 which were revealed in exhausted T cells by Woosung Chung et al. from studying single-cell RNA-seq data of tumour cellular landscape, which is highly unlikely in case of bulk RNA/cDNA isolations from heterogenous populations of metastatic and non-metastatic cancer cells, eventually boosting development of anti-metastatic therapies [98–101]. Single cell transcriptomics data used to characterize glioma as a systemic brain disease found single tumor cells detected throughout the brain exhibiting power of SCA in deciphering disease profiles [102]. Also, single-cell DNA sequencing has made significant discoveries of mutational evolution in tumors revealing diverse phenotypes of cancer cells [103]. Circulating tumor cells (CTCs) are found to be majorly responsible for metastasis of cancer and are in extremely low abundance *in vivo* [104]. Diagnostic devices powered by microfluidic devices have exhibited the ability to detect CTCs, offering early diagnosis and increased survival rates in cancer patients [105]. Microfluidic technology in single-cell isolation has become popular and is employed in various types of cancer research. One such study reports detecting metastatic cancer cells based on their phenotypic traits such as size and deformability, where cancer cells are captured using microchannels of various dimensions in a polydimethylsiloxane (PDMS) device with an efficiency of more than 78%. Microfluidic technologies like these can improve primary cancer diagnostics while eliminating the need for preprocessing and labeling of cells in blood [106]. In other studies, detection of cancer cells was achieved by analyzing the output signal from captured/uncaptured cells which were surface functionalized with various components including anti-EpCAM (epithelial cell adhesion molecule) and anti-EGFR (epidermal growth factor receptor) RNA aptamers which bind with upregulated proteins specific to cancer cells, nano roughness and other ligands specific to cancer cell adhesion [59,107,108]. Other technique like formalin-fixed, paraffin-embedded-disaggregation for intracellular signaling in single epithelial cells from tissues (FFPE-DISSECT) preserved the native environment of cells on which time-of-flight mass spectrometry (CyTOF) was applied to study single cell activities such as signaling pathways which can count in pathologically significant signals from smaller populations of cells unlike western blot or ELISA which consider homogeneity in cell populations. Using FFPE-DISSECT Simmons et al. found differential

tumor necrosis factor-alpha associated signaling in intestinal enterocytes, goblet cells, and enteroendocrine cells and reduced differentiation in colorectal cancer specimen compared to normal colons [82]. Techniques like FFPE-DISSECT can be applied to study cellular landscapes in tissue microenvironment especially in various tumors to assess drug responses and help reveal resistant subpopulation of cells. Various cancer disease progression factors including cellular metastasis & stemness profiles, intratumor heterogeneity, DNA methylation and many other unknown mechanisms can be investigated with the latest SCA techniques.

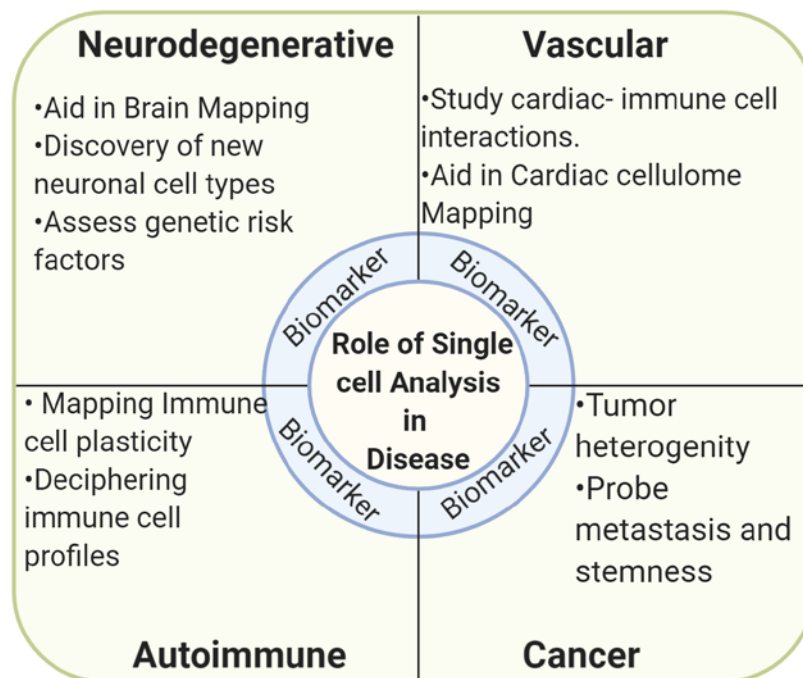


Figure 3. SCA role in study of diseases.

4. SCA in cell adhesion study

Cell adhesion plays a vital role in signaling, tissue development and maintenance. “Cell adhesion model” predicts that the number of chemical bonds to the surface is proportional to how much of cell sticks [109]. Cell differentiation, cell cycle, cell migration and cell survival are in part stimulated by signals involving cellular adhesion [110]. Hence, there is a need to study the cell adhesion mechanisms at a single-cell level to learn more about the interconnectivity between adhesion-based signaling and cellular response. Irregularities in cell adhesion mechanisms can be a prominent event for the onset of diseases including arthritis, cancer, osteoporosis, and atherosclerosis [110–118]. A diverse system of transmembrane glycoproteins called Cell adhesion molecules (CAM) including I-CAM, V-CAM, selectins, integrins, etc., has been linked in cell-cell and cell-extracellular matrix adhesion and is responsible for the development and maintenance of the tissues. Research shows that the disruption of epithelial cellular interactions with extracellular matrix (ECM) leads to apoptosis, a phenomenon known as “anoikis” [119]. Tumor cells have gained the potential to resist the anoikis process by over

expression of caspase-8 inhibitor FLIP which is involved in death receptor pathways and bcl-2 family based anti-apoptotic protein in mitochondrial pathways, promoting metastasis of cancer [120]. Cell adhesion molecules are highly involved in tumor metastasis and other cellular invasiveness such as T cell infiltration in endothelial dysfunction which is indicative of aging effects [113,121]. An in-vitro assay of a tumor spheroid in a 3D collagen matrix was performed to study the stress induced by fibrosarcoma cells on matrix gel; revealing a large contractile force on the matrix before invasion [122]. Single-cell force spectroscopy and single-molecule force microscopy used in combination with Atomic force microscope (AFM) and optical microscopy can probe the forces between cell-substrate, cell receptor and ligand, revealing the cell elasticity and other receptor binding behaviors towards various ligands like proteins, improving design of molecular target moieties in drug delivery [123]. Towards measuring cell adhesion, Sifeng Mao et al. developed live single-cell extractor (LSCE) which uses trypsin in between vertical channels to separate cells and the outlet channel for trypsin on the chamber is aspirated to remove the adhered cell and the time taken for the cell to detach is calculated towards the cell and biomaterial adhesion strength [124]. SCA method like LSCE can investigate better cell culture biomaterials, modified ECM-cell interactions and cell heterogeneity profile based on biomaterials unlike conventional methods which use cell spreading and viability for analyzing cell-adhesion behaviors. This research display that nano and micro scale topographical features from substrates have a strong influence on cell adhesion, alignment, morphology and contact guidance which in part dictates the cell behavior. Studies on naturally occurring nano-textured surfaces (NTS) such as dragonfly wings have shown anti-bactericidal properties, where strong adhesive forces are seen between the NTS and E.coli producing a fatal amount of shear on cell membrane when bacteria moves, killing the bacterial cell [60]. Also, NTS has seen to have profound effect on how metastatic cancer cells change their morphology in comparison with non-metastatic cells; the shape change in metastatic cancer cells is higher in NTS than smooth substrates showing the topological significance in cell-substrate interactions [59]. Therefore, SCA techniques for examining cellular responses for novel nano and microscale topographical substrates is noteworthy to assess the cellular behavior at single cell level to find significant molecular interactions [61,125]. The neurons in the brain are interconnected presumably by cell adhesion molecules to form the circuits which are responsible for various neurological signaling pathways of the human body and many related cell surface molecules such as cadherin, neuexin, immunoglobulins and leucine-rich repeat protein superfamilies' are currently investigated in the context of neural circuitry [126]. Overall, studying cell adhesion at single-cell-level can help us understand cell signaling events, phenotype and morphology and develop diagnostics such as cancer detection based on surface or substrate adhesion-based cell capture.

5. SCA role in precision medicine

Variable treatment responses is of major concern as it drives up costs in drug development, hospital care and other healthcare related proceedings. SCA driven tools and techniques developed and the data generated from them are facilitating clinical studies such as pre-implantation diagnosis [127], immune and disease cell responses to drugs [62], *in vitro* models to assess response of immune cells e.g. CAR-T cells before transplantation [56], etc where heterogenous cell populations are considered unlike conventional bulk populations, improving response rate, reducing drug development costs and inherently improving economics of healthcare. Hui Dong et al. developed a SCA technique, where a

microchip was used to culture human breast cancer cells (MCF-7) and treated them with drug reagents like methyl methane sulfonate, docetaxel, colchicine followed by cell lysis, amplification of specific genes to study the genotoxicity induced from the drug reagents; exhibiting the possibility of reducing reagent consumptions and instrumental cost per reaction [62]. Similarly, Yu Du et al. developed a new *in vitro* model of a liver sinusoid, mimicking the *in vivo* structure and function using four kinds of functional hepatic cells and a layered structure [63]. Devices like these can be used to understand the organ functionality, cytotoxic metabolism and other drug induced responses in desired cell populations. In another intriguing approach to analyze mechanical biomarkers, acoustic actuation was employed for drug-screening platforms to screen multiple single cells at the same time [128]. This technique works by creating acoustic streaming resulting in hydrodynamic forces which deform the cells and the deformability factor generated can be used as a mechanical biomarker in screening drug responses and identifying highly deformative and invasive metastatic subpopulations in cancer cells; which can serve as an early diagnosis marker. Another study where single cell cultures were analyzed with techniques such as RNA FISH and ATAC-seq to assess whether cancer cells gain resistance through genetic inheritance or epigenetic reprogramming during drug treatment; and it was discovered that small fraction of cell subpopulations with high resistance markers undergo further epigenetic reprogramming such as loss of SOX10 and activation of other new signaling pathways which made them shift from transient transcriptional state to a stable resistant state exhibiting a multi stage drug resistance attainment in cancer cells; these new approaches can create new clinical models to design personalized drug dosages [129]. Also, investigating cell-cell signaling, especially immune cells which are linked with major diseases and aging, could help in designing treatment strategies; for instance, Sarkar et al. developed a microfluidic single step loading droplet array platform where they encapsulated individual single human T cells and dendritic cell in nanoliter droplets with a facility to activate cells after encapsulation for e.g. in this study ovalbumin activated dendritic cell and T-cell dynamic interactions including intracellular cytoplasmic calcium levels, an indicator of in T cells activation and cell-cell contact cytotoxicity of T-cells in various environments [56]. SCA platforms like these could be employed for drug screenings, development of vaccines and study complex cell-cell interactions and their dynamics of mapping in various cell contact profiles eventually improving cellular engineering. Large datasets from “omics” studies can provide information in creating novel algorithms and experimental models to be used for Insilco drug design and modeling which can empower pre-clinical and personalized medicine.

6. Current challenges in SCA

In single cell analysis, studies are in the scale of micro and nano dimensions where obtaining better resolution of the images and control over molecular size is quite challenging. When cells are studied *in vitro* they are not in their natural habitat and we lose the capability to study their original characteristic features which demand for better *in vivo* mimicking study models for SCA. SCA omics studies produce a massive amount of data which consists of biologically and pathologically significant signals along with noise. Better theoretical models and sophisticated algorithms in computational programs are required for efficiently detecting those important signals, such as sub-cellular population data, and relevant genomic transcript data from the noise produced in omics'. A more detailed review of challenges in single cell analysis is laid out by Guo-Cheng Yuan et al. [130].

7. Future directions

Validation of cell types and lineages and significant molecular entities identified through single cell experimental procedures is of critical importance in creating standard logistics which can help in further clinical discoveries and downstream applications. There is a need for development of lineage tracing technologies which have a potential of mapping disease development and progressions in creating prediction models to track various stages of an unknown pandemic and epidemic disease outbreaks based on shared genetical and epigenetical transcripts, for instance, Hayes J et al. were able to identify novel microRNA signatures in a varying severity of glioblastoma in patients helping them to build a survival risk score which may be further employed in designing therapies [131]. Leonard D. Goldstein et al. report a novel nanowell-based single-cell RNA sequencing system called ICELL8 microchip which has the capacity to sequence 1300 single cells and characterize cell types [132]. Devices like ICELL8 accompanied with powerful computation tools can help profile gene expressions of various species cells concurrently enabling comparative disease profile analysis across species revealing common pathologically significant mechanisms and their innate therapeutic pathways which may help in pre-clinical drug screenings in our mammalian counterparts [133]. Genetic mosaicism appears to be a rule more than an exception and every cell in the human body have their own personal genome, i.e. a unique genomic signature [127]. Unique genetic signatures of cells, cellular molecular profiles and other microenvironment information produced from SCA techniques can be used to develop nanoparticles/nanobots [134] with biophysical actuating and sensing capabilities like cellular barcoding [135] or acoustofluidic coating [31] incorporation to image or sense for diagnostic and therapeutic cues *in vivo*. In single cells the volume of molecules available to fit into a model is of very low concentrations and significant mechanisms at cellular level might be due to the chance encounter of reactant molecules, To avoid false positive data, there is a need for more robust molecular probes and efficient data interpretation of information generated from SCA. One such approach via stochastic modeling of the whole biochemical signaling network of single cell can enable us to understand the molecular evolution of reactive species and can help build single cell atlas. For instance, Khatibipour et al. have developed a software, jacobian-based method (JacLy) in an attempt to analyze metabolic interactions and fluctuations from small networks in metabolome data [136,137]. Similarly, cellular data modeling such as the one developed by Mitra Mjtahedi et al., can be deployed in clinics for having estimations on the production of cell colonies of the reprogrammed cell and their therapeutic metabolites improving product economics in pharamaceutical industry [9]. Although SCA techniques have a great potential to analyze with molecular resolution, there is a need to develop more physiology-driven models mimicking organ microenvironment where pathologically significant cells and other biological entities like bacteria, virus can be studied more robustly [63] to investigate their genotype and phenotype changes in an relevant physiological environment than convetional *in vitro* 2D cell cultures [138]. And enormous data generated from the Omics study of single cells usually contains noise signal which may hinder the ability to find physiologically significant signals. Development of efficient measurement models is needed to identify significant signals from noise e.g. Bayesian probabilistic models integrated into single-cell RNA-sequencing measurements have the power to detect differential expression signatures and identify cellular subpopulations despite the noise at single cell level [139]. Interdisciplinary oriented research teams in single cell analysis can improve research output as SCA deals with various sciences including biochemistry, molecular biology, computational biology,

biophysics, etc. Finally, we are in an era of studying single cell biology and understanding the most fundamental mechanisms that characterize life and its irregularities which lead to aging and disease.

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Conflict of interest

The authors declare no conflict of interest for the contributions in this manuscript.

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