

# Application of single-cell sequencing in human cancer

Mattias Rantalainen

Corresponding author: Mattias Rantalainen, Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Nobels Vag 12A, Stockholm 17177, Sweden. E-mail: mattias.rantalainen@ki.se

## Abstract

Precision medicine is emerging as a cornerstone of future cancer care with the objective of providing targeted therapies based on the molecular phenotype of each individual patient. Traditional bulk-level molecular phenotyping of tumours leads to significant information loss, as the molecular profile represents an average phenotype over large numbers of cells, while cancer is a disease with inherent intra-tumour heterogeneity at the cellular level caused by several factors, including clonal evolution, tissue hierarchies, rare cells and dynamic cell states. Single-cell sequencing provides means to characterize heterogeneity in a large population of cells and opens up opportunity to determine key molecular properties that influence clinical outcomes, including prognosis and probability of treatment response. Single-cell sequencing methods are now reliable enough to be used in many research laboratories, and we are starting to see applications of these technologies for characterization of human primary cancer cells. In this review, we provide an overview of studies that have applied single-cell sequencing to characterize human cancers at the single-cell level, and we discuss some of the current challenges in the field.

**Key words:** single-cell; sequencing; cancer; personalized medicine

## Introduction

The clinical importance of comprehensive molecular phenotyping of cancer tumours is increasing with the advent of precision medicine [1], which aims to provide tailored treatment to individual patients based on their molecular phenotype. DNA sequencing and RNA sequencing (RNA-seq), together with many other molecular profiling technologies, enable comprehensive molecular phenotyping of tumours and have been applied to characterize many cancer types in projects like the cancer genome atlas [2, 3]. Sequence-based molecular phenotyping reveals quantitative information on a multitude of molecular levels, including data on somatic and germ-line single-nucleotide variation, copy number variation (CNV), gene fusions, DNA methylation and gene expression variability. Conventional molecular profiling is based on an average molecular phenotype from a large population of cells (often described as a 'bulk' sample within the context of single-cell studies), which has proven useful in many applications. However, substantial loss of information occurs through

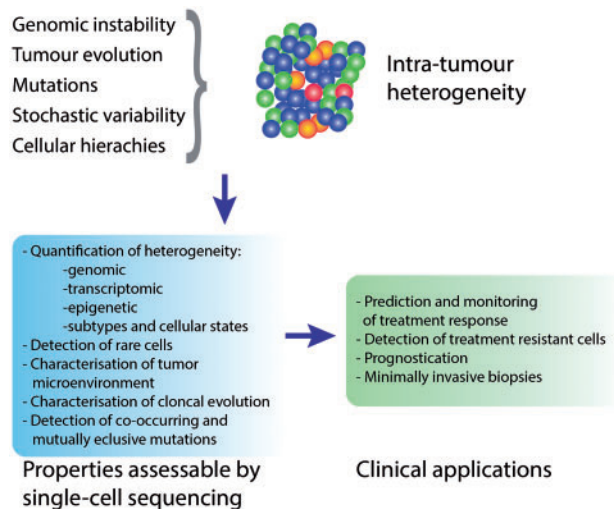
averaging over the molecular phenotype of individual cells. Single-cell molecular phenotyping has the capability to generate high-resolution molecular phenotype information and provide means for quantitative analysis of several key properties of tumours, including intra-tumour heterogeneity, cellular composition (cell types), cellular hierarchies and cell states. It is likely that single-cell molecular phenotyping will replace bulk average molecular profiling in many cancer research and clinical applications in the future.

Cancer is a disease with inherent heterogeneity [4–6] caused by multiple factors, including intra-tumour evolution, cellular plasticity [6] and multiple sources of stochastic variability (Figure 1). Chromosomal instability, which leads to intra-tumour heterogeneity, is associated with poor patient outcomes [7], and cancer patients with larger proportion of subclonal mutations have also been observed to have a higher chance of relapse [8]. A key challenge in cancer treatment is detection of rare subpopulations of cells that have the potential to develop resistance to therapy. Such subpopulations of cells can be either subclones or subpopulations of cells that through stochastic

**Mattias Rantalainen** is an assistant professor at Karolinska Institutet in Sweden. His research is focused on development and application of statistical and bioinformatic methodologies to address problems in cancer genomics and personalized medicine.

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**Figure 1.** The role of single-cell molecular phenotyping in characterization of tumour heterogeneity and in clinical applications. Multiple molecular mechanisms and environmental factors lead to intra-tumour heterogeneity. Single-cell molecular phenotyping enables characterization of many aspects of intra-tumour heterogeneity and has the potential to be applied to generate clinically relevant information.

processes or cellular plasticity can adapt to changing selective pressure from treatment or other environmental factors.

Single-cell sequencing of tumour cells are improving our ability to characterize intra-tumour heterogeneity, and it is likely that intra-tumour heterogeneity will prove to be clinically relevant in the context of precision medicine [4, 5]. Tumour heterogeneity can arise on multiple levels, through clonal evolution and through heterogeneity in cell states that are the effect of dynamic molecular phenotypes, including epigenetic and transcriptomic effects, or a combination of multiple molecular levels. Quantitative characterization of tumour heterogeneity in particular, including detection of rare subclones of cells with possible drug resistance potential, has the potential to be translated to the clinic in the future. There are multiple clinically relevant applications where single-cell sequencing and information on tumour heterogeneity is likely to be of importance, including prediction of treatment response, prognosis, monitoring of disease progression, prediction of treatment effect and detection of emerging drug resistance (Figure 1). Single-cell sequencing can also be applied for molecular phenotyping of circulating tumour cells (CTCs) [9], i.e. cancer cells disseminated into the bloodstream, with samples collected through minimally invasive liquid biopsies.

There are many reviews covering different aspects of single-cell technologies and applications, including reviews focused on single-cell genomics [10–14], cancer genomics [10, 11, 15–17], CTCs [18, 19], tumour heterogeneity [6] and single-cell analysis [10, 20, 21]. In this review, we focus on providing an overview of studies, where single-cell sequencing has been applied for characterizing primary human cancer cells, and we discuss some of the current challenges for single-cell molecular phenotyping of patient-derived cancer cells.

### Single-cell sequencing of primary cancer cells

Development of technologies for single-cell isolation, whole-transcriptome or whole-genome amplification (WGA) together

with next-generation sequencing provides the foundation that has enabled the emergence of single-cell sequencing. Generation of single-cell sequencing data from primary human cancer cells can be described through a set of fundamental process steps (Figure 2): (1) sample acquisition from patient; (2) creation of single-cell suspension; (3) temporary storage; (4) isolation of single cells and library preparation; (5) sequencing; and (6) bioinformatic and statistical analyses. Owing to logistical challenges when working with clinical samples, there are typically delays in sample processing (Step 3). This logistic delay can often be avoided when working with model systems (animals or cell lines), while it remains a reality in many studies based on patient material (e.g. biopsy) that is collected at a clinic.

### Sample handling

In studies based on clinical samples, e.g. biopsies or surgically removed tumours, it is essential to ensure that the molecular integrity of the samples is preserved until molecular phenotyping. To accomplish this, samples either have to be processed immediately at time of collection or a method that allows preservation of the molecular integrity has to be applied. Immediate single-cell sequencing of fresh samples is often challenging to implement because of separation in physical location between specialized laboratories and clinic (Figure 2, Step 3). If samples are collected for later molecular phenotyping, a single-cell suspension is generated followed by application of a preservation method compatible with downstream molecular profiling. Evaluation of a few methods for temporary storage of samples for single-cell sequencing applications, including cryopreservation [22] (DNA- or RNA-seq), methanol fixation [23] (DNA- or RNA-seq) and CellSave [24] (DNA sequencing), has recently been reported. Single-cell sequencing of cryopreserved cells [22], as well as methanol fixed cells [23], revealed high transcriptomic concordance with fresh cells. Recently, a method for preservation of cells for single-cell RNA-seq without chemical crosslinking or freezing [using CellCover (AL Anacyte Laboratories UG), DNA RNA preservation] was also applied in a single-cell RNA-seq study [25]. Clinical samples are routinely prepared as formalin-fixed, paraffin-embedded (FFPE), which limits the opportunity for single-cell sequencing, especially in respect to RNA sequencing. Martelotto et al. [26] evaluated a method for single-cell whole-genome copy number profiling in FFPE material based on isolation of intact nuclei using fluorescence-activated cell sorting (FACS) sorting. Results of this study suggested that CNV profiles from FFPE material can be comparable with single-cell fresh-frozen material [26]. For CTC analysis either positive or negative selection, or a combination thereof, has to be applied to isolate the CTCs from blood. Liquid biopsies (e.g. blood samples) have to be kept in a state where RNA and DNA are not degraded before molecular phenotyping. In a study evaluating three different available preservatives [K3EDTA, Cell-Free DNA BCT (BCT) and CellSave (Cellsearch)], BCT and CellSave provided the best preservation of CTCs, while BCT provided the better preservation of RNA in comparison with K3EDTA [24]. Further development and evaluation of protocols for sample preservation methods compatible with single-cell DNA- and RNA-seq are necessary to enable wider application of single-cell sequencing to characterize clinical samples. Large collaborative efforts, for example ‘the human cell atlas’ [27], will most likely contribute to the development and systematic evaluation of improved sample handling protocols, which is essential to enable large-scale application of single-cell profiling.

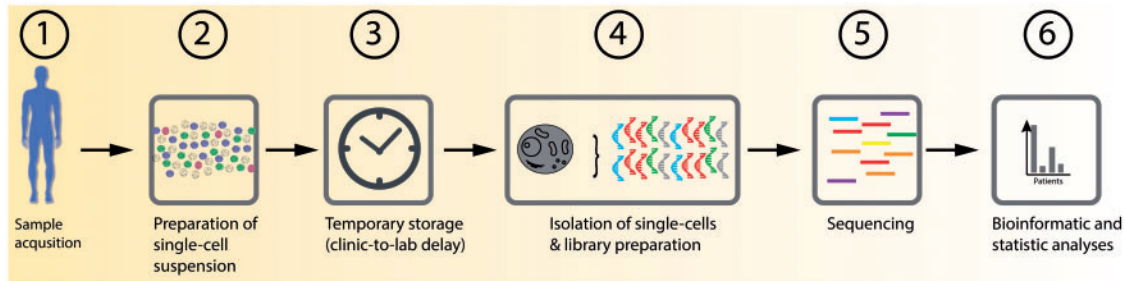


Figure 2. Overview of the process of applying single-cell sequencing to patient-derived tumour samples.

### Single-cell isolation

Single-cell sequencing typically requires a suspension of individual cells as starting material. In situations where single cells from solid tissues are to be profiled, dissociation of the tissue into a cell suspension has to be accomplished as a first step, followed by isolation of the individual cells. Techniques for single-cell isolation from cells in suspension have been reviewed extensively before and include FACS (DNA- or RNA-seq), microfluidics (DNA- or RNA-seq), droplet-based capture (RNAseq), Laser Capture Microdissection (DNA- or RNA-seq) and manual selection (DNA- or RNA-seq) [14, 17, 28, 29]. More recently, a novel microwell-based approach [25] (RNAseq) and methods based on combinatorial indexing [30, 31] (DNA- or RNA-seq) have also been proposed, offering cost-effective high-capacity methods for single-cell isolation and library preparation. The different methodologies differ in respect to fundamental physical principles and the maximum number of cells that can be captured. The choice of method for single-cell isolation depends on the context and objective of the study. Single-cell analysis of CTCs provides an attractive surrogate biopsy of primary or metastatic tumours, as liquid biopsies can be collected in a minimally invasive procedure through a conventional blood sample [32]. CTCs are present in exceptionally low frequency in the blood ( $\sim 1$  of  $10^9$  blood cells), making efficient enrichment and capture methods important. Many methods and strategies have been reported for CTC isolation and reviewed elsewhere [19, 33–35]. Cellsearch (Veridex) is one of the most widely applied platforms for CTC enumeration and capture of CTCs [36]. Cellsearch is based on positive selection using antibodies against EpCAM and cytokeratins (positive markers) and against leukocyte antigen CD45 (negative marker) together with a nuclear dye (4',6-diamidino-2-phenylindole). Cellsearch enrichment together with single-cell isolation using DEPArray (Silicon Biosystems) has been applied in multiple studies [37, 38]. Additional CTC enrichment and capture methods include Magsweeper [39], flow cytometry [40], microfluidic devices [41, 42], HD-CTC [43], MINDEC [44], Rosettesep (STEMCELL Technologies Inc.), EPIC CTC platform [45] and CTC ichip [46].

### Single-cell sequencing

There are now multiple methods available for DNA and RNA sequencing in single cells. Single-cell sequencing protocols all require amplification of the genomic DNA or complementary DNA, in the case of RNA-seq, before preparation of sequencing libraries. Single-cell DNA sequencing has proven to be more challenging compared with RNA-seq, as each cell contains many RNA molecules, but only two copies of DNA. Currently, single-cell RNA-seq is more established than single-cell DNA

sequencing, with a more diverse set of methods available for single-cell RNA-seq. Studies applying single-cell RNA-seq typically include larger numbers of cells (hundreds or even several thousand cells in recent studies) compared with those that focus on single-cell DNA sequencing.

WGA of the single genome copy is currently necessary for single-cell DNA sequencing, and ideally, the amplification procedure should have minimal biases and sequence errors. There are multiple methods for WGA with different limitations and performance in respect to genome coverage and uniformity. The most commonly applied methods are polymerase chain reaction (PCR)-based (DOP-PCR) [47, 48], isothermal amplification (MDA) [49], hybrid methods (MALBAC) [50], together with proprietary methods including GenomePlex WGA4 (Sigma-Aldrich) based on PCR amplification of randomly fragmented genomic DNA. The relative performance of these methods has been evaluated [51–53], and commercial kits, including AMPLI1, MALBAC, Repli-G and PicoPlex, for single-cell exome sequencing were evaluated in [54]. WGA methods were also previously reviewed from a comparative perspective [14]. Furthermore, Baslan *et al.* [55] proposed a modified DOP-PCR method with improved performance and cost-effectiveness for single-cell CNV profiling. Zahn *et al.* [56] described a direct library preparation method for single-cell genome sequencing for CNV analysis, which display higher degree of uniformity compared with WGA-based methods.

Application of single-cell RNA-seq requires amplification of the RNA transcripts before sequencing. Methods for single-cell RNA-seq can be categorized in methods that perform full-length transcript amplification (Smart-seq, Smart-seq2) [57, 58] and those with a 5' [59] or 3' [60] bias; see [61, 62] for an overview of single-cell RNA-seq methods. In addition to DNA and RNA sequencing, novel methods for single-cell sequencing of additional molecular levels are emerging, including accessible DNA regions (ATAC-seq [63, 64]), chromatin conformation [65], epigenetic modification [66, 67], simultaneous DNA and methylation [68] and simultaneous sequencing of DNA and RNA [69]. Methodologies for single-cell DNA and RNA sequencing have been reviewed previously in [12–14, 17, 70, 71].

### Single-cell analysis

Common objectives in bioinformatic and statistical analyses in single-cell cancer studies are analyses of intra-tumour heterogeneity, molecular subtyping at the single-cell level, detection of rare cell types, mutation detection, CNV profiling and lineage inference. To gain the most out of the single-cell sequencing studies, specific models and methods should be used in some applications instead of methods developed for analysis of conventional bulk average profiles. Single-cell RNA-seq data in

particular have distinctly different distributional properties compared with conventional bulk average RNA-seq data, including substantially zero-inflated expression distribution and latent variability because of, e.g., cell cycle effects [72] (RNA). WGA typically leads to data with limited genome coverage, and allelic dropout leads to loss of one or both alleles at some locations during amplification. An increasing number of specialized methods for analysis and modelling of single-cell data are available, including methods for rare cell detection [73–75] (RNA), differential expression [76–78] (RNA), pathway analysis [79] (RNA), imputation [80–82] (RNA), heterogeneity [78, 79] (RNA), lineage inference [83, 84] (DNA), pseudo-time-ordering [85–87] (RNA), clustering [80, 88] (RNA), dimensionality reduction [89–91] (RNA), modelling of latent factors [72] (RNA) and quality control [92] (RNA). Reviews of bioinformatic and statistical methods for single-cell analysis are also available [10, 20, 21].

## Applications of single-cell sequencing for molecular phenotyping of human cancer cells

Here, we provide an overview of studies that apply single-cell sequencing to characterize primary human cancer cells, while studies based on cell lines, xenograft models and primary cultures are not included in the current survey. Most of these studies are of small size, particularly in respect to the number of patients. The number of single-cells from each patient is also limited in many of the studies, although the more recent studies include larger number of cells [93–95], reflecting the ongoing technology development in the field. It is evident that single-cell sequencing has been applied across a range of different cancer disease using both DNA- or RNA-seq, and addressing a range of general and specific research questions, some of which are outlined in the introduction. Studies are summarized in two tables, Table 1 including single-cell sequencing of primary cancer cells, and Table 2 including studies focused on single-cell sequencing of CTCs. We comment on some of the key studies below.

### Single-cell sequencing of primary cancer cells

CNV profiles of primary single cells from two breast cancer patients were reported in an early landmark study demonstrating feasibility of the method and revealing distinct clonal subpopulations of cells as well as concordant CNV profiles between primary tumour and metastasis [96]. In another study, CNV profiles were generated from breast cancer tumours and disseminated tumour cells (DTCs) from bone marrow [97], and CNV profiles were compared between primary tumour or lymph node metastases and DTCs, revealing concordance of CNV profiles in 53% of identified DTCs and allowing for phylogenetic analysis and ability to determine the origin of DTCs [97]. Gawad *et al.* [98] applied targeted single-cell DNA sequencing to study childhood acute lymphoblastic leukaemia (ALL) in 1479 cells from 6 patients, which allowed them to gain insights into clonal evolution, development of ALL and determine co-occurring mutations. Single-cell RNA-seq was applied in a study of metastatic melanoma based on 4645 cells from 19 patients using fresh material and single-cell isolation by FACS [95]. The cells profiled in this study included cancer cells as well as stromal, immune and endothelial cells, thus allowing for characterization of the tumour microenvironment in addition to analysing inter- and intra-tumour heterogeneity. Interestingly, subpopulations of cells with expression of genes indicative of resistance to

targeted therapies were identified. Interactions between cancer cells and tumour microenvironment were also investigated [95]. In another study focused on glioblastoma patients, the RNA of single cells was sequenced, revealing intra-tumour heterogeneity in respect to established molecular subtypes suggesting possible effects on prognosis [99]. Tirosh *et al.* [94] used single-cell RNA-seq to characterize fresh single-cells from human oligodendroglioma patients. They characterized intra-tumour heterogeneity and found that cancer cells mainly belonged to two subgroups defined by their expression profiles, together with a smaller third subgroup of undifferentiated cells with stem cell-like expression profiles, which also had a high proliferative potential [94].

### Single-cell sequencing of CTCs

Liquid biopsies provide means for minimally invasive collection of biopsies from cancer patients that allow for single-cell sequencing of CTCs. Single-cell sequencing of CTCs has been applied across a range of cancer disease (Table 2), but most of the sequencing-based CTC studies are too small to be able to draw conclusions regarding clinical outcomes. In a study of CTCs from lung cancer, it was found that the CNV profile was concordant with metastases in the same patients, while single-nucleotide variation was found to be heterogeneous from cell to cell [109]. Analysis of CNV profiles and genomic instability in CTCs from metastatic castrate-resistant prostate cancer demonstrated ability to detect key alterations with clinical relevance, including loss of *PTEN* and amplification of androgen receptor (*AR*) [117]. Single-cell RNA-seq was also used to profile prostate cancer CTCs, which revealed significant within-patient heterogeneity, including expression of *AR* splice variants (*AR-v7*) associated with resistance against anti-androgen treatment [113]. Results from a study based on cultured CTCs from breast cancer patients have indicated heterogeneity in respect to *HER2* status, including spontaneous and dynamic interconversion (i.e. cell-state plasticity [6]) between *HER2+* and *HER2-* status in cell populations, which may contribute to development of drug resistance [118]. CTCs have been demonstrated to be predictive of treatment response in both breast and prostate cancers [119–121].

## Discussion

Single-cell sequencing is revolutionizing cancer research by providing a significant step forward in respect to the resolution at which the molecular phenotype of tumours can be characterized. Intra-tumour heterogeneity is common in many cancer diseases and related to treatment response, progression and survival outcomes. Intra-tumour heterogeneity can only be fully characterized at the single-cell level. Methods for single-cell isolation and DNA and RNA sequencing are now well established and further improvements and novel methodologies are continuously being developed. Single-cell methods for collection of additional molecular levels beyond DNA and RNA are also being developed and so are single-cell multi-omics methods where multiple molecular levels are profiled in the same cell, providing unique opportunity to generate comprehensive molecular phenotypes of tumours with single-cell resolution.

Study design is of key importance in single-cell cancer studies. However, there are few studies to date that cover aspects of study design in single-cell studies. The cost is approximately proportional to the (number of patients) \* (number of cells per patients) \* (number of sequencing reads per cell), but depending on application, these factors should be carefully considered.

Table 1. Overview of studies applying single-cell sequencing to characterize primary human cancer cells

Tumour type	Analyses	#Patients	#Cells tot	Molecular level	Single-cell isolation	Amplification method	Year	Reference
Breast cancer	Clonal subpopulations, comparative analysis of primary cancer cells and metastasis	2	200	CNV	FACS	GenomePlex WGA4	2011	[96]
Clear cell renal cell carcinoma	Intra-tumour heterogeneity, tumour evolution	1	25	WES	Manual	REPLI-g Mini Kit (MDA)	2012	[100]
Essential thrombocythemia	Mutation profiling, clonal evolution	1	90	WES	Manual	REPLI-g Mini Kit (MDA)	2012	[101]
Childhood ALL	Analysis of clonal structure, detection of subclones	6	1479	Targeted DNA sequencing	Fluidigm C1(microfluidic)	GenomePhiv2.MDA kit	2014	[98]
Glioblastoma	Intra-tumour heterogeneity	5	430	RNA-seq	FACS	SMARTer Ultra Low RNA Kit	2014	[99]
Neuroblastoma	DTCs, mutation profiling	10	144	Targeted DNA sequencing	Silicon Biosystems DEPArray	AMPLI1	2014	[37]
Breast cancer	Comparative analysis of primary cancer cells and DTCs	6	63	CNV	Manual	GenomePlex WGA4	2016	[97]
Hepatocellular carcinoma	Genomic, epigenetic and transcriptional heterogeneity	1	25	RNA-seq, DNA methylation, CNV	Manual	scTrio-seq	2016	[102]
Metastatic melanoma	Intra-tumour heterogeneity, cell states, tumour microenvironment	19	4645	RNA-seq	FACS	Smart-seq2	2016	[95]
Oligodendroglioma	Heterogeneity, cellular architecture, cancer stem cells	6	4347	RNA-seq	FACS	Smart-seq2	2016	[94]
Acute myeloid leukaemia	Intra-tumour heterogeneity, expression profiling, mutation profiling	1	20	RNA-seq	Fluidigm C1(microfluidic)	SMART-Seq v4 Ultra Low Input RNA kit	2017	[103]
Breast cancer	Intra-tumoural heterogeneity, tumour microenvironment	11	515	RNA-seq	Fluidigm C1(microfluidic)	SMARTer Ultra Low RNA Kit	2017	[104]
Colorectal cancer	Heterogeneity, subtyping	11	590	RNA-seq	Fluidigm C1(microfluidic)	SMARTer Ultra-Low RNA Kit	2017	[105]
Glioblastoma	Inter- and intra-tumour heterogeneity	3	305	RNA-seq	Fluidigm C1(microfluidic)	SMARTer Ultra-Low RNA Kit	2017	[106]
Gliomas	Cellular hierarchies, tumour microenvironment, intra-tumour heterogeneity	16	14 226	RNA-seq	FACS	Smart-seq2	2017	[93]
High-grade serous ovarian cancer	Intra-tumour heterogeneity	1	92	RNA-seq	Fluidigm C1(microfluidic)	SMARTer Ultra-Low RNA Kit	2017	[107]

Note: WES, whole-exome sequencing.

Table 2. Overview of studies applying single-cell sequencing to characterize circulating tumour cells (CTCs)

Tumour type	Analyses	#patients	#Cells tot	Molecular level	CTC enrichment	Single-cell isolation	Amplification method	Year	Reference
Colorectal carcinoma	Mutation profiling	2	14	Targeted DNA sequencing	CellSearch	Manual	GenomePlex	2013	[108]
Lung cancer	CNV profiling, SNV profiling	11	72	WES, WGS, CNV	CellSearch	Manual	MALBAC	2013	[109]
Breast cancer	CTC heterogeneity	18	221	Targeted DNA sequencing (PIK3CA)	CellSearch	Silicon Biosystems DEPAArray	Ampli1	2014	[110]
Breast cancer	CTC heterogeneity,	12	26	Targeted DNA sequencing (PIK3CA and TP53)	FACS	FACS	Ampli1	2014	[111]
Breast cancer	CTC heterogeneity, comparison with tumour biopsy	2	11	Targeted DNA sequencing (TP53)	CellSearch	Silicon Biosystems DEPAArray	Ampli1	2014	[112]
Prostate cancer	Intra-tumour heterogeneity, AR, treatment resistance	13	77	RNA-seq	CTC-iChip	Manual	mRNA-Seq whole-transcriptome analysis of a single cell	2015	[113]
Prostate cancer	CTC heterogeneity, SNV, structural rearrangements, comparative analysis between CTCs and primary tumour cells	1	4	WGS	NanoVelcro chip	Laser capture microdissection	MDA	2015	[114]
Breast cancer	CTC heterogeneity, mutation profiling	4	24	Targeted DNA sequencing	CellSearch	Silicon Biosystems DEPAArray	Ampli1	2016	[115]
Multiple myeloma	Monitoring of treatment response, mutation profiling	9	335	Targeted DNA sequencing	RosetteSep	Manual	REPLI-g Mini Kit (MDA)	2016	[116]

Note: WES, whole-exome sequencing; WGS, whole-genome sequencing.

Recent developments in single-cell sequencing have increased the number of cells that can be isolated and sequenced [122]. However, in studies with larger number of cells, fewer sequencing reads from each individual cell are typically collected, thus limiting the sensitivity of molecular phenotype data acquired from the cells. To evaluate association with molecular data and patient outcomes, larger number of patients will have to be included in studies. Detection of rare cell types, or rare cell states, requires profiling of larger number of cell from each patient. The amount of sequencing reads collected from each cell is related to the sensitivity in detecting and quantifying the molecular phenotype, and although different cell types can be correctly classified with relatively limited RNA-seq data from each cell (<50 000 s reads/cell), more sequencing reads (up to several million reads/cell) will be required to determine more subtle differences that reflect cell states in the transcriptomic profile, which is expected to be relevant in molecular phenotyping of cancers. At the time of writing no systematic evaluation of these different study design factors have been reported, while we expect the trade-off between these factors to be central for the success of future studies. To determine a reasonable study design, it is advisable to apply power calculations, especially when patient outcome analyses (e.g. time-to-event analyses) are a primary objective. Deciding the number of single cells to profile in each patient will be directly related to the degree of heterogeneity and the desired power to detect and profile rare cells or cell states. However, such information might not be readily available, and in such situation, a pilot study can provide the necessary information to determine a suitable study design.

Single-cell methods will undoubtedly become an increasingly important tool for basic cancer research using model systems (e.g. cell lines, animal models, xenograft models). However, applications in human cancers research and precision medicine are now emerging and provide opportunity to understand how heterogeneity and tumour evolution contribute to clinically relevant outcomes, including probability of treatment response, progression and survival outcomes. At the moment, liquid biopsies, including single-cell sequencing of CTCs, represent a technology that probably has the highest chance of rapid translation to the clinic. Single-cell sequencing has so far generated promising results in relatively small studies of patient derived cancer cells, and the next step will be to initiate larger studies with more patients to evaluate to what extent molecular phenotype data with single-cell resolution provide advancement in prognostication, prediction of treatment response or other relevant outcomes.

Many challenges remain to be addressed before single-cell sequencing will be routine in clinical cancer research and translated to the clinic. Sample handling and requirement of fresh cells are major obstacles in studies based on patient-derived tumour cells that have to be overcome. Development of methods and protocols that enable preservation of cells for later single-cell sequencing is essential to scale up studies based on clinical samples. Although currently available methodologies have enabled successful application of single-cell sequencing to a wide range of problems in human cancer genomics, further development of methodologies and technologies in multiple areas is required to advance single-cell profiling in cancer research. Efficient isolation of single-cells is still dependent on substantial infrastructure that is rarely available to individual laboratories. Development of efficient yet affordable methods for single-cell isolation would enable wider uptake of single-cell methods, and there are already some advances in this direction [25]. Isolation of CTCs also remains a challenge, and methods

with improved capture efficiency would broaden the potential applications of non-invasive liquid biopsies of cancer patients and CTC sequencing. Development of new methodologies for single-cell multi-omics profiling, enabling measurements of multiple molecular levels in the same cell, has the potential to substantially improve our capability to characterize molecular mechanisms of cancer. In respect to analyses of single-cell RNA-seq data, there are few methods and models available that account for technical noise and zero-inflated distributions for cancer-specific analyses, including detection and classification of cell states or subtypes at the single-cell level. There is also an emerging need for novel methodologies and models that allow for analysis of multi-omics single-cell data, as these methods are starting to become available. Currently, the cost of large-scale application of single-cell sequencing can be prohibitive; however, we anticipate that with the currently ongoing rapid development of both single-cell and sequencing methodologies costs will inevitably come down over time and open up many new application areas.

## Conclusions

The field of single-cell sequencing is rapidly developing and has over the past few years reached a maturity level, where clinically relevant information (including intra-tumour heterogeneity, development of treatment resistance and tumour evolution) can be collected through profiling of single-cells from cancer patients. Many of the fundamental objectives of cancer precision medicine (prediction of treatment response, prognostication, detection of treatment resistance) are possible to address at a higher resolution with single-cell methods compared with conventional bulk average molecular phenotyping. Therefore, it is highly likely that single-cell molecular phenotyping will supersede bulk average profiling in the future in many application areas. We are now just starting to see the beginning of this trend. The next step in application of single-cell methods in the study of human cancers is to initiate studies that include larger patient cohorts; larger numbers of single-cells and that also consider clinical outcomes.

### Key Points

- Intra-tumour heterogeneity is an inherent property of many cancers and may play a central role in respect to clinical outcomes
- Single-cell sequencing technologies provide means for high-resolution molecular phenotyping of large numbers of individual cancer cells and enable characterization of intra-tumour heterogeneity
- Most single-cell studies of human cancers to date include few patients, which limit the opportunity to investigate effects on clinical outcomes.
- Larger studies that include more patients are now needed to establish potential associations between the unique information captured by single-cell sequencing and clinically relevant outcomes

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