Clinical Genomic Analysis and Diagnosis —Genomic Analysis *Ex Vivo*, *in Vitro* and *in Silico*

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Abstract   Seven years ago, I systemically reviewed single cell techniques with genomic and proteomic analyses which was called Single-Cell Genomic Analysis. After many years of arduous work, single cell techniques with downstream genomic and proteomic analysis have been applied to clinical fields including molecular pathology, molecular genetics, forensic medicine and biomarker discovery. On top of that, dynamic cell-sorting technique combined with downstream cell culture and genomic analysis of stem cell for regeneration medicine and cancer stem cell for differentiation have also been greatly developed in clinical fields. More importantly, tissue level sampling with *in silico* analysis has been applied in therapeutic targeting for advanced neoplastic disease. Recent development in sorting homogeneous cells *in vitro* (or single cells technique), *ex vivo* (dynamic analysis or small number of cell culture with downstream genomic analysis) and *insilico* (tissue level sampling with *in silico* analysis) have allowed physician scientists with a choice to select one of these above techniques with genomic analysis to apply to their clinical research fields. To fully understand these modern techniques, this manual will review recently developed methods or clinical genomic analysis *in vitro*, *in silico* and *ex vivo*. In the review paper, I will also introduce how to utilize these techniques in different clinical fields. The manual will also address some of the challenges for clinical genomics analysis and diagnosis due to mixed cells from clinical specimens.

Keywords   Genomics Analysis, Clinical Genomics Diagnosis, Single Cell Diagnosis, Single Cell Genomics Analysis , Diagnosis, Primary Cell Culture, Biomarker Discovery, Therapeutic Targeting

1. Introduction

The mixed cell population in clinical samples can mask the actual results of genetic diagnosis and genomics analysis. In order to overcome the challenges in purity and limited cell counts after initial purification, single cell technique, or similar techniques, have now being widely developed for over a decade. One of the most common is single cell PCR[1]. Due to advancements in techniques in amplifying genomic DNA/RNA and signal magnifying for protein from small number of cells, these techniques, including genomics and proteomics are now widely being employed in the clinical fields[2]. Based on research and development for genomic analysis from clinical samples, physicians and scientists are studying faster/purer techniques, such as laser capture microscopy, along with downstream genomic analysis to study clinical specimens. The single-cell harvest obtained from glass slides combined with downstream genomics and proteomics have been developed in molecular pathology, molecular genetics and forensic medicine[3]. Some call the

technique, cytogenomics[4]. The technique is named as *in vitro* analysis because the procedure defined as **in vitro** (Latin: *within the glass or glass slide*) is performed outside the living organism. On the other hand, dynamically pure cell-sorting technique combined with *ex vivo* culture with downstream genomic analysis (such as stem cell for regeneration medicine and cancer stem cell for inducing differentiation) has also been tremendously developed[5]. This technique is known as **ex vivo** because the measurements are done in an artificial growth environment (outside the organism) with minimum alterations of the natural conditions. Moreover, after Dr. Schmid first used "micro-dissection *in silico*" to analyze gene expression profiles to uncover biomarkers from clinical specimens[6], tissue level sampling by *in silico* analysis is now increasingly being applied in genomic analysis of heterogeneous cells from clinical tissues[7]. Here the technique is called as **in silico** genomic analysis due to the performance *via* computer or program simulation, as an analogy to the Latin phrases *in vivo* and *in vitro* refer to experiments done in living organisms and outside of living organisms, respectively. In order to incorporate all the clinical genomic techniques by using these three methods, *in vitro, ex vivo* and *in silico*, I will refer to clinical genomic analysis as genomic analysis and diagnosis by *in vitro, ex vivo* and *in silico*. Genomic

analysis *in vitro,* used in molecular pathology and molecular genetics from pretreated tissue[8], combines cell isolation and genomic analysis from clinical specimen. Genomic analysis *ex vivo*, encompass three processes, 1) living cell separation, 2) culture and 3) downstream genomics analysis as well. Now, FACS/MACS for living cell separation can be combined with fixed cell immunohistochemical staining (IHC)[9] and laser micro-dissection technique (originally only used for fixed cell) can be utilized in living cell with downstream cell culture[10]. Genomic analysis *in silico*, direct tissue-level specimens for genomic analysis, in which several modules in bioinformatics are able to identify specific genomic profile in the mixed tissue level, Here, in order to better understand clinical genomic analysis and diagnosis from clinical specimens, I will compare genomic analysis among *intro*, *ex vivo* and *in silico* for different clinical specimensand different purposes. Based on their conditions and purposes, clinical scientists can decide which way is the best genomic analysis for their specimens.

2. Clinical Genomic Analysis by *intro, ex vivo,* and *in Silico*

In my previous review paper[11], it was shown that the flow-cytometric cell sorting, magnetic cell separation (FACS/MACS) and laser-based micro-dissection of tissues provide the basic methods to isolate similar cells to study gene expression profiling from clinical specimens. FACS isolating technique for cells in solution labelled with

fluorescent signals can sort these cells with a specific biomarker such as CD3/CD4/CD8 for lymphocytes and CD133/CD34 for stem cells and cancer stem cells (CSCs). At present, multi-colored fluorescence-activated cell sorters (multi-coloured FACS) can selectively separate and collect homogeneous cells with identical phenotypic features in a collection tube so that FACS can increase its ability to study gene expression profiling in a given cell type[12]. In the other fields, laser micro-dissection technique to isolate cells on glass slides labelled with fluorescent signals or markers can sort clinical cells that rely on specific mRNA/protein biomarkers and morphology change such as tumor cells or cancer stem cells. The characteristics of laser micro-dissection have allowed us to quickly study a given cell *in vivo* localization and to analyse the cell's microenvironment *in vivo*[13].

Relied on research and development, the techniques for sorting single-cell or a small number of homogeneous cells from clinical specimens have been developed and categorized by the methods, purposes and functions described in Table 1.

Here I will introduce each method,in details, along with this advantage and disadvantage in the following order: (1) genomic analysis *ex vivo* covering cell-sorting with downstream culture *ex vivo* and genomic analysis; (2) genomic analysis *in vitro* including treated cell (fixed and stained cells) and un-treated cell and (3) direct genomic analysis *in silico* for clinical specimens.

Table 1.   Cell preparation of clinical genomic analysis

Category	Methods	Cell situation	Clinical application
In vitro	Laser capture microscopy	fixed cells	A. Clinical diagonsis I. molecular pathology II. forensic medicine B. Clinical treatment I. therapeutic target II. personalized therapy
	1). FACS 2). MACS 3). single cell manipulation	living cells	A. therapeutic target B. personalized therapy
In/ex vitro	1). FACS 2). MACS 3). single cell manipulation	living cells	A. Stem cell B. Cancer stem cell C. Lymphocytes
In silico	1). Cell methods A. PCA B. Cluster C. SOM	tissue levels	A. therapeutic target B. personalized therapy
	2). molecular level A. supervised learning B.time course	tissue levels	A. therapeutic target B. personalized therapy

*In vitro means the technique of performing a given procedure in a glass or glass slides environment outside of a living organism; *in vivo* is experimentation using a whole or living organism; *ex vivo* is experiment done for tissue in an artificial environment outside the organism; *in silico* means an expression or experiment "performed on computer or via computer simulation".

2.1. Genomic Analysis *ex vivo*

Cell culture from clinical specimens is different from cell lines. Cells that are cultured directly from clinical specimens are known as primary cells. The primary cells isolated from tissues can be cultured *ex vivo* or *in vivo* environments. The primary cell cultures can be often used in lymphocytes, cancer stem cell, stem cells and primary cancer cells[14].

Along with the research of stem cells for regenerative medicine, the study of cancer stem cells for biomarker discovery and the application of T-cell for passive and active immunotherapy as shown in Table 2, stem cells, cancer stem cells, and T-cells harvested from clinical specimens with genomic analysis can play an increasingly important role in biological and medical fields. Although *ex vivo* differentiation of stem cells into special somatic cell types

such as neurons and myocytes has a well-established model, long-term culture of *ex vivo* autogenously adult stem cells so far have not been completely successful. The extremely low number of these cells in primary hematopoietic organs and the lack of good culture systems that support proliferation of undifferentiated stem cell have influenced their biological research and clinical application. In 1999, we reported cancer cell cultured in high-dose radiated mice to increase the cell number to study the cell characteristics[15]. Encouragingly, now repopulating capacity of *ex vivo* culture of hematopoietic stem cells has also largely been reported, which will indicate a good future for regeneration medicine[16]. In addition, *ex vivo* culture and long-term storage of induced pluripotent stem cells (IPS cells) have been tremendously reported.

Table 2. Difference between *ex vivo* and *in vitro* cell-sorting for clinical genomic analysis

Methods	Difference of cell preparation	Cell process	Clinical application
ex vivo	dynamic process for *ex vivo* culture cell	1. Fixed cells 1). ICC 2). DNA/RNA FISH 2. Living cells 1). FACS 2). MACS 3). single cell manipulation	A. cancer stem cell I. biomarker discovery in stages II. therapeutic targeting in stages B. stem cell I. control and tageting cell differentation in different stages C. lymphocyte activating and quiescence control in tumor and immune response
in vitro	one-time process for cells on slides/tube	1. Fixed cells 1). ICC 2). DNA/RNA FISH	A. cancer stem cell I. biomarker discovery II. therapeutic targeting B. stem cell for biomarker discovery

LCM means laser capture microscope; ICC is immunocytochemical staining; FISH is Florescent In situ hybridization.

Table 3. Clinical genomic analysis *In vitro**

Methods	Cell preparation	Clinical application
Laser capture microscopy	1. Labelling cells 1). ICC 2). IHC 3). DNA FISH 4). RNA FISH 2. Morphology 1). tumor cell 2). forensic cell	A. Clinical diagnosis I. molecular pathology II. molecular genetics III. forensic medicine B. Clinical application I. biomarker discovery II. therapeutic target III. personalized therapy
non-laser capture microscopy	1. Living cell labelling A. FACS B. MACS C. Single cell manipulation 2. FRET (mRNA)	A. Cancer stem cells/tumor cells I. therapeutic target II. personalized therapy B. Stem cell differentiation control C. Lymphocyte activating and quiescent

LCM means laser capture microscope; IHC is immunohistochemical staining; FISH is Florescent In situ hybridization and FRET means fluorescence resonance energy transfer.

2.2. Genomic Analysis *in vitro*

Since 1976, laser micro-dissection has been increasingly applied in different fields as shown in Table 3. Currently, three microdissection methods have been routinely employed. Those are (1) laser-assisted mechanical tissue micro-dissection[17], (2) laser pressure catapult micro-dissection[18] and (3) laser capture micro-dissection[19]. Most laser-based microdissections require tissue pretreatment such as fixation, dehydration and staining. The application of these techniques requires fully consideration to downstream work such as DNA array CGH, mRNA microarray and proteomics. The advantage of laser-based microdissections can be combined with Ab-based immunohistochemical/immunocytochemical (IHC/ICC) or DNA FISH and RNA FISH staining to increase the cell specificity from their biomarker staining. In these several years, following developmental requirement of cancer stem cells for biomarker identification and therapeutic targeting and the application of stem cells for regeneration medicine, laser-based micro-dissection will be faced with two large developments: (1) as indicated above, process from cultured living cells, which can continue culture for downstream genomic analysis; (2) combined with automation system, whose process can be used for high-throughput screening such as for biomarker discovery[20].

Cell-sorting technique by FACS and MACS is also quickly developed. With FACS technique development of multi-colored fluorescence-activated cell sorters and increasing antibody products, the FACS technique can play a much more important role in cell-sorting technique for genomics and proteomics analysis of clinical specimens[21]. Nowadays, two fields are being quickly developed, the one is based on labelling mRNA inside cells to sort cells by florescent-labelled mRNA and the other one is combined with automation system, the purpose is high throughput screening to discover new biomarkers[22].

Due to impurities of FACS and MACS in clinical specimens, we have added a single cell manipulation to increase the purity after MACS or FACS process so downstream-cells can provide more accurate data in genomic signature analysis and diagnosis for patient's therapeutic targeting[23].

2.3. Genomic Analysis *in silico*

In the clinical field, clinical specimens are often and directly frozen due to requirements of pathological processes. If the specimens are processed at the tissue level for microarray by total mRNA, array CGH by total genome DNA and proteomics by total protein, genomic analysis *in silico* is an exclusive way for the analysis of genomic data because the genomic data at the tissue level are mixed with genome from different cells[24]. According to our experiences, at least three ways can be used for genomic analysis for heterogeneous cells: hierarchical cluster, principle component analysis (PCA), and self-organizing map (SOM). Hierarchical clustering can be quickly processed due to its usage of similar expression patterns for cell groups. Principle component analysis (PCA) is primarily aimed at finding complex relationships between variables in a dataset so it has been extensively applied in biomarker discovery of known cells from mixed-cells tissues. This feature helps us to study variables and factors that are not correlated to each other[25]. The self-organizing map (SOM) is a powerful tool for grouping and visualizing high-dimensional complex data which can be applied for a two-dimensional plane[26]. Following extracting data from mixed cells of clinical specimens, we have routinely employed two of the three platforms to analyse data from the mixed cells as shown in Table 4. If genomic data from the clinical specimens do not contain a series of control samples to process micro-dissection *in silico*, we can use clinical bioinformatic module combined with biological or medical biomarkers corresponding this disease to analyse the data, such as using "supervised leaning" protocol.

Table 4.   Clinical genomic analysis *in silico*

Methods	Platform	Feature
Cell levels	1. Cluster analysis	Quickly processed for cell groups
	2. PCA	Finding complex relationships from mixed-cells tissues
	3. SOM	Grouping and visualizing high-dimensional complex data
Molecular level	1. Supervised learning	Based on specific biomarkers
	2. Time-course	Based on molecular change

Cluster analysis is Hierarchical clustering; PCA is Principle component analysis and SOM is Self-Organizing maps
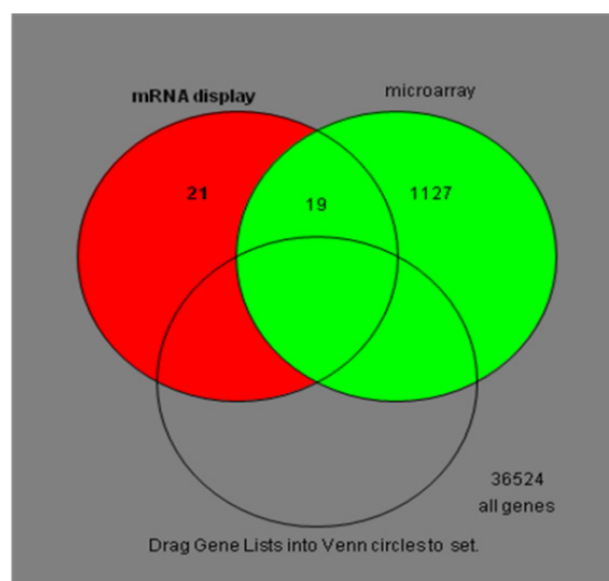

Figure 1.   Relationship between genomic analysis *in vitro* (single-cell genomics or single-cell mRNA display) and genomic analysis *in silico* (microarray in tissue level)

We have studied a set of genomic data (genomic analysis *in silico* via genomic analysis in vitro, or single cell genomic analysis) from similar sample as Figure 1, both will produce

accurate intersection results. The results show that genomic analysis *in silico* can mine three digit genes. This method is highly sensitive that this can be used to uncover genome profiles; on the other hand, single cell genomic analysis can detect genes, which is so specific that this can be utilized to confirm the genomic profile.

3. Application of Clinical Genomic Analysis

3.1. Regeneration Medicine

A major issue in regenerative medicine is the cell sources used to rebuild damaged tissues. Although many questions are still unanswered, most physicians and scientists nowadays have reported that regeneration in humans from the body's own tissues is feasible. Regeneration is a regulative developmental process ubiquitous across all human organs. It functions throughout the life cycle to restore the normal function of cells, tissues, organs, appendages and whole organisms.The regenerative capability is absent or low in some cells such as hepatocytes, myofibers, osteocytes, and most neurons[27]. We know that three sources of cells, induced pluripotentstem cells(IPS cells), embryonic stem cells (ESCs) and adult stem cells (ASCs) can be used for cells and/or organs regeneration for damaged tissues. ESCs are *in vitro* cultivated pluripotent cells derived from the inner cell mass (ICM) of the embryonic blastocyst. ES cells can be differentiated into representative derivatives of all three embryonic germ layers (endoderm, ectoderm and mesoderm) in both *in vitro* and *in vivo*[28].Adult stem cells also can multiply to regenerate the definitive cells to replace damaged tissues. They have been found in normal scaring after injury, such as in myocardium, pancreatic islet cells, spinal cord and retina tissues[29]. Because stem cells (ESC and ASC) have high growth and self-renewal capacity, several papers and protocols have been successfully reported to use the cells into the therapeutic possibilities, such as diabetes, mycardiac infarction and Parkinson's disease.

Genomic analysis *ex vivo* can play a critical role in proliferation and differentiation of stem cells for regenerative medicine, such as dynamic monitoring genomic profile for a small amount of cultured cells. After we understand the genome and the corresponding pathway from the primary cells in a differentiating period, we can select corresponding growth factor or compound to control cell proliferation and to target differentiation based on the genomic analysis.

3.2. Clinical Diagnosis

3.2.1. Single Cell Diagnosis *Via* Single Cell Genomic Analysis/Diagnosis

The pathology diagnosis relies on cell morphology and cell arrangement such as tumor cell diagnosis based on the cell morphological change and infiltrating into normal tissue. The diagnosis of molecular genetics and cytogenetics prefers chromosome structure for analysis. Following the development of single cell techniques, a new term, "Single Cell Diagnosis", has emerged in molecular pathology and molecular genetics/cytogenetics[30]. As shown in Table 5, single cell diagnosis can be utilized for several fields, such as pathology, genetics and microbiology. Now, this technique can be used in many clinical laboratories, for example, surgical specimens from operation, biopsy specimen from internal medicine (especially in haematology), blood samples from paediatrics (perinatal diagnosis), obstetrics/gynaecology and psychiatrics as well[31].

Table 5. Single cell diagnosis *via* single cell genomic diagnosis

Specimens	Methods	Application
DNA level	PCR	Genetic disease (mutation/SNP and LOH)
	Array CGH	Tumor disease (mutation/SNP and LOH)
		Other diseases
mRNA level	rtPCR microarray	Biomarker discovery Therapeutic targeting
Proteinlevel	Western blot Proteomics	Biomarker discovery Therapeutic targeting

Single cell genomic analysis and diagnosis extended from single cell technique have some advantages over single cell diagnosis, for instances, (1) following genomic analysis from single cell harvest, it can convert pathological changes into biomarker discovery for genetic and tumor disease; (2) along with genomic analysis, single cell genomic analysis and diagnosis can linkpathogenesis of disease and therapeutics so single cell genomic analysis and diagnosis can be used for personalized medicine; (3) single cell genomic analysis and diagnosis can extend beyond genetic disorder and cancer disease to diagnosis of several other diseases.

3.2.2. Forensic DNA analysis *via* Single Cell DNA-profiling

The characteristics and the inheritance of autosomal and sex chromosomes are the basis to determine DNA typing in forensic medicine. Forensic DNA analysis, such as, restriction fragment length polymorphisms (RFLP), short tandem repeats (STR), variable number tandem repeats (VNTR), and mitochondrial DNA has been routinely applied into forensic laboratory. Along with human genome decoded and development of forensic-DNA techniques, the application of forensic-DNA analysis have extended from a blood sample into DNA trace specimen such as single cells or small number of cells. In RFLP technology, the polymorphism can be read by HPLC and DNA-Sequencer in a specific gene or non-coding DNA sequence in a individual person[32]; STR analysis combined with PCR technology with fluorescent labels, can automatically detect at a single

cell level and analyzed the DNA genomic profile for a great amount of specimens[33]; because VNTR are inherited from the individual's parents,, it also can be used for a single cell DNA-profiling[34]; Since mitochondrial DNA is passed from one generation to the next solely through the maternal line of a family, By comparing the mitochondrial DNA (mDNA)from samples of maternal families and maternal relatives, forensic scientists are able to search and demonstrate a specimen trace in a maternal family[35,36].

3.3. Clinical Treatment

3.3.1. Biomarker Discovery

Early diagnosis and treatment is a key factor required to reduce the mortality and morbidity of all types of diseases, especially for tumor disease. Unfortunately, currently available cancer and genetic screening tools (CT, X-ray, mammography and invasive needle or surgical evaluation for cancer and genetic disease) are not sensitive enough for early detection of the diseases thus most tumor diseases cannot be treated at an early stage. Moreover, it is imperative to develop non-invasive technique for diseases, such as tumors that can be distinguished between patients with and without cancer, as well as stages of cancer. At present, genomic and proteomic technologies have rapidly been involved in cancer research[37, 38, 39]. Genomic technologies have allowed us to monitor thousands of gene expression profiles and evaluate functions of candidate genes to obtain a global view of cancer tissue. Proteomic techniques have also allowed us to understand proteins and their modifications. Despite its remarkable usefulness, microarray and proteomics techniques all have technical limitations because of cell purity or limited cell number after initial purification from clinical specimens so that, if we do not have a rational module for clinical genomic analysis, clinical genomic data cannot clearly define some specific biomarkers and the accompanied therapeutic targeting.

In order to address the important question, here I introduce characteristics, advantages and disadvantages of the genomic analysis *ex vivo*, *in vitro* and *in silico* for application of clinical specimen.

The most useful protocol uncovering biomarker is *in vitro* genomic analysis, such as single-cell manipulation by laser capture microscopy (LCM) along with genomic analysis. If we have known cell morphology in a tumor disease and/or information from cancer cell such as biomarker or telomerase activity, we can pick the cells with the morphological feature and enzyme activity, and then process genomic analysis from the harvested cells. After *in vitro* genomic analysis, we will uncover very specific biomarkers[40]. If the cells are located in liquid such as leukemia cells in bone marrow or peripheral blood, living cell-sorting such as FACS/MACS with downstream genomic analysis allows us to discover specific biomarkers from clinical specimens.

Additionally, genomic analysis *ex vivo*, or living cell-sorting technique with the downstream cell culture to increase cell number or understand differentiating stage and then genomic analysis can help us to discover some biomarkers at different stages of cancer[41]. As mentioned above, genomic analysis *ex vivo* has been employed into cancer stem cell.

Recently, genomic analysis*in silico* has been extensively utilized to discover tumor biomarker and biomarkers related with genetics. As shown in our result[42], genomic analysis *in silico* can screen or mine some very specific biomarkers in some diseases. Because of mixed different cells from clinical tissue remaining in the mined gene profile, current results from bioinformatic analysis still need further experiments, such as rtPCR or Western blot to confirm the mined gene profiles. Genomic analysis *in silico* is a good substitute method if the specimens have not been processed for micro-dissection *in vitro* orcell-sorting *in/ex vivo*.

3.3.2.Therapeutic Targeting

Therapeutic targeting is a specific treatment for clinical diseases. In details, after genomic analysis along with pathway information and linking drug database to provide information, the corresponding drugs will directly target the explored therapeutic protein and nucleic acid for the targeted disease. A necessity of therapeutic targeting, clinical genomic analysis, nowadays, has been quickly applied into tumor disease, immune disease and genetic disease. Major developments are in two fields: A. therapeutic targeting for cancer stem cell; B. individualized therapy for metastatic tumor, immune disease or genetic disease.

Now we all know, small populations of cells, called cancer stem cells (CSCs) located in tumor tissues play an important function in the development and progression of the disease[43,44,45]. It is also thought that CSCs drive the metastatic spread of cancer. CSCs are able to resist conventional therapies so that the disease is difficult to be completely eradicated. If therapeutic targeting identification can uncover some specific proteins or nucleic acid of CSCs, the selective targeting of CSCs will offer a new paradigm in both cancer diagnostics and therapeutics. According to current report, more than 30 CSC R&D program are subject to several clinical research to discover drugs or compound. Now, most CSC R&D program is being taken forward to large pharmaceutical companies[46,47].

In clinical fields, personalized medicine (or individualized therapy), one of special therapeutic targeting, is going to extend into different clinical diseases. Personalized medicine is a new medical model to be directly defined as physicians enable tailored approaches to prevent and care for individual patient relying on genomics and proteomics. It is often defined as "the right treatment for the right person at the right time." All examples of successful personalized treatments require a rational clinical genomic analysis. Based on research and development of clinical genomic analysis, we have successfully established a bioinformatics module for personalized therapy, that is, following genomic signature mined by genomic analysis *in silico*, quantitative pathway

analysed by topology process, a specimen with no small cell lung cancer (NSCLC) was used to mine significant targeting, finally uncover therapeutic targeting with linking drug database for the special treatment[48].

4. Conclusions of Clinical Genomic Analysis

Seven years ago, I describe single cell genomic techniques based on experiences in our laboratory and other laboratories. After further research and development of clinical genomic techniques with maturity in amplification for genomic DNA/RNA and signal magnification for protein for the past several years, many new genomic techniques have been developed into clinical specimens, for instances, dynamically single-cell or small number of cell-sorting techniques with downstream *ex vivo* culture to increase the cell number or differentiation into a special type of cells and then genomic analysis; moreover, genomic analysis at tissue level with *in silico* analysis has also be quickly developed. These new clinical genomic analyses have been extended from molecular pathology, molecular genetics/cytogenetics, forensic medicine into stem cells for regenerative medicine and tumor cells and/or cancer stem cells for biomarker discovery and therapeutic targeting and into T-cell for adoptive immunotherapy.

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