REVIEW ARTICLE

The Evolution of *In Situ* Genetic Technology

Andrew Ford, Charmaine Brown, Elizabeth Caver, Chen-Hsiung Yeh

Circulogene Theranostics, Birmingham, USA

ABSTRACT

In situ genetic technology was historically developed and mainly focused on detection purpose, allowing specific nucleic acid sequences to be visualized in morphologically preserved tissue sections. With the synergy of genetics and immunohistochemistry, *in situ* detection can correlate microscopic topological information with gene activity at the transcriptional or post-transcriptional levels in specific tissues. Furthermore, its resolution allows spatial distribution of nucleic acid products to be revealed in a heterogeneous cell population. The newest member to the franchise of *in situ* genetic technology is a direct-on-specimen enrichment methodology specifically for cell-free DNA liquid biopsy. Contrary to *in situ* detection, this in-well *in situ* innovation tackles the very first sample preparation step to reduce material loss, thereby improving overall sensitivity. Genomic nucleic acids purified from specimens have been proven to be time consuming and suffered from damages and losses; the evolution of *in situ* genetic technology offers a powerful tool for precision functional genomics, enabling cross-check between *in vitro* and *in vivo* findings. It further opens the door to ultimate genetic engineering *in situ*.

Key words: Cell-free DNA, direct-on-specimen enrichment, *in situ*, liquid biopsy

IN SITU POLYMERASE CHAIN REACTION (PCR)

Since the discovery of DNA structure in 1953, a great deal of effort has been devoted to detecting specific target sequences. The breakthrough technique, PCR, conventionally performed in a tube, permits the amplification of single-copy mammalian/viral gene from purified DNA/RNA. However, the prerequisite nucleic acid extraction step precluded localization of the target to its specific cell of origin, and in many instances, this is critical information. *In situ* PCR combines the sensitivity of PCR with spatial localization of the target to monitor the appearance of specific gene or transcript in the tissue sections. The use of *in situ* amplification to histologically detect and localize low-copy genes or transcripts offers several advantages over traditional methods. In particular, PCR affords both increased sensitivity and the capability, by the judicious design of primers, to detect transcripts of discrete members of gene families.[1] The principle of this method involves tissue fixation (to preserve the cell morphology) and subsequent treatment with proteolytic digestion (to permeabilize cells and provide access for the PCR reagents to the target DNA/RNA). The target sequences are amplified and then detected by standard immunocytochemical protocols.[2] The ability to identify individual cells, expressing or carrying specific genes of interest in a tissue section under the microscope provides a visual account of gene expression and regulation, and enables the determination of various aspects of normal versus pathological conditions, or latent versus active pathogen infection. For example, simple and important data such as the percentage of a given cell type that contains the target of interest cannot be obtained because of the obligatory destruction of tissue. In the case of virus infection, although HIV-1 RNA can be routinely detected from lymph nodes, in seropositive, asymptomatic patients, it is very important

Address for correspondence:
Dr. Chen-Hsiung Yeh, Circulogene Theranostics, 3125 Independence Dr., Suite 301, Birmingham, AL 35209, USA.
Tel: 1-205-234-0128. E-mail: cyeh@circulogene.com

to identify which of the many different cell types that reside in this tissue are reservoirs of the virus before the development of AIDS.

All of the *in situ* PCR techniques attempt to create sufficient DNA targets within the cell, which can either be detected directly or following an immunohistochemical step. For successful application of the techniques, the aim is to achieve a fine balance between adequate permeabilization of cells (allowing access of amplification reagents) and maintaining localization of amplified product within the cellular compartment while preserving tissue/cell morphology.[3-5] To do this, the techniques require specific thermal cycle changes to occur at the individual cell level, resemble to that which occurs in solution phase PCR. However, unlike solution PCR, an exponential increase in the amount of amplified product is never achieved. Instead, linear amplification occurs in most situations because of the relative inaccessibility of nuclear target sequences and inefficiency of amplification on cellular compartmentation. *In situ* PCR amplification is viewed as a controversial procedure owing to the variability in detection of amplified products. Thus, it is no surprise that most laboratories consider it to be an Research Use Only tool for investigational studies. However, the potential applications of this technology to all facets, including gene therapy, provide enough stimulus to warrant further validation of its applications. In the near future, as the number of gene therapy protocols continues to increase, so too will the need for determining not only which cells have received the newly introduced gene but also which of these cells are expressing this gene. In this respect, gene therapy trials will certainly benefit from *in situ* PCR to localize a new gene and/or gene product within specific cell types.

IN SITU HYBRIDIZATION (ISH)

ISH technique originally used autoradiographic labeling to map both repetitive and low-copy DNA sequences, i.e., specific annealing of a labeled nucleic acid probe to complementary sequences in fixed tissues followed by visualization of the location of the signal. Although it was very sensitive, problems associated with this original technique include short half-life, safety problem, and long-exposure time which hindered its widespread use in DNA hybridization. To overcome these problems, non-isotopic ISH was developed with further progress in this field, led to chromogenic *in situ* hybridization,[6] and the current standard of care test of fluorescence *in situ* hybridization (FISH).[7] FISH offers the possibility to specifically mark individual chromosomes over their entire length or defined chromosome regions in metaphase chromosomes and interphase nuclei preparations. It is a method that can be used to detect small structural aberrations (e.g., deletion, amplification, and duplication) that are not visible in traditional karyotyping. The target and reference probes were labeled with different fluorophores when a sample has a deletion; only one target signal can be seen instead of two. When a case has a duplication, three target signals can be observed instead of just two.[8] FISH can also specifically interrogate breakpoints of chromosomal rearrangements. More than one target probe can be applied to the same tissue section to detect different nucleic acid targets. Using different detection systems with each probe, resulting in different color end products, and visualization of the different nucleic acid targets can be achieved.[9,10] Whereas FISH is faster with directly labeled probes, indirect labeling offers the advantage of signal amplification using several layers of antibodies, and it could, therefore, produce higher signal-to-noise ratio.[11] In general, FISH can be performed on all available tissues with cells containing nuclei. Cytological preparations, as well as cultivated cell material (chromosomes in metaphase or interphase nuclei) and uncultivated single cell preparations, can be processed.

The development of ISH technologies has provided us with a wealth of clinical information regarding the chromosomal aberrations, locations, and expression patterns of genes at single cell level. For discovery research, complete gene expression profiles of single cells will provide a new level of insight into the correlation of gene expression patterns with particular biological phenotypes. This will be particularly important in studies of molecular diagnostics as well as development and disease progression, where complicated; finely demarcated gene regulation programs are in play. Accordingly, the transcriptional profiling of whole tissues can, therefore, be misleading when ascribing a function to a gene and caution should be observed when interpreting expression data from tissue samples containing more than one cell type. Awareness of such issues has led to the extensive application of ISH that has allowed researchers to examine the expression of specific genes in specific cell types.

IN SITU SEQUENCING

It would be ideal to define genetic functionality using high-throughput molecular profiling *in situ* with high-resolution imaging. However, such approaches represent a massive experimental undertaking and produce only an average view of tissue-specific gene expression.[12,13] So far, this approach is limited by the number of spectrally distinct fluorophores and the optical diffraction limit of microscopy.[14] To overcome these challenges, a method of highly multiplexed subcellular RNA sequencing *in situ* called fluorescent *in situ* sequencing (FISSEQ) for detecting tissue-specific gene expression, RNA splicing, and post-transcriptional modifications was developed.[15,16] This novel technology allows investigators to both, pinpoint the location of thousands of RNA molecules at once in intact cells, and determine the sequence of each transcript.

The method is carried out with enzymes that copy each mRNA into a complementary strand of DNA and multiplying that DNA strand many times to create millions of replica DNA molecules *in situ*. Then, with the application of four different fluorescent dyes - one for each of the DNA's four bases - a sequence of flashing colors reveals each replica DNA's exact sequence under a super-resolution microscope. To better capture and differentiate the fluorescent signals from the background that often densely packed with replica DNA structures, a technique called partition sequencing that assigns each replica DNA clone a barcode based on its base sequence was developed, allowing to only analyze a fraction of the replica DNA beads at a time. FISSEQ potentially can be applied in disease diagnostic as well as therapeutic areas. For example, in cancer research, it might lead to earlier diagnosis, helps track how gene mutations impact local cancer invasion and metastasis, better define responses to modern targeted therapies, and uncover new drug targets. In addition, the method could also provide valuable insights into how the spatial and temporal expression and regulation of distinct mRNAs relates to cell differentiation and tissue morphogenesis during embryonic development. *In situ* sequencing could potentially achieve both high spatial resolution and high throughput, but current *in situ* sequencing techniques are still in its infancy stage of development. Inefficiency and variation in amplification, ligation, sequencing, and barcode readout, all need to be further improved before commercialization.

IN SITU CIRCULATING CELL-FREE DNA ENRICHMENT

Current progress in cancer theranostics is limited by the difficulty of collecting repeat invasive biopsies and obtaining high-quality tumor material. A major innovation that will dominate the personalized oncology sector for years to come is "liquid biopsy," driven by key advantages that these tests can offer over traditional tumor biopsy. The facts include the ability to provide clinically actionable information in a non-invasive, faster, and cheaper way, the potential to provide real-time longitudinal surveillance related to the efficacy of a therapeutic regimen, patient response, evolving drug resistance, and disease recurrence.[17] These characteristics enable the selection of best-targeted therapy specific for each patient based on tumor clonal evolution without delay. Liquid biopsy cell-free DNA (cfDNA)-based *in vitro* diagnostics will become a complementary tool for oncologists, gap filling the limitation of tissue biopsy, and completing tumor profiling for personalized medicine for the treatment of cancer. Consequently, it is expected that the number of "liquid" samples that clinical laboratories will manage would be significantly higher compared to tissue samples. In such scenario, highly automated and high-throughput system for cfDNA isolation needs to be implemented. Most importantly, circulating cfDNA concentration is usually very low and highly fragmented with short peak fragment of around 170 bp and its multiples which appeared to correspond to di- and tri-nucleosomal DNA. Consequently, current cfDNA extraction methodology all suffered from significant sample loss due to poor silica-binding efficiency, which can profoundly affect downstream analytical accuracy.[18]

We analyzed tumor genome using cfDNA prepared by an *in situ* approach (LIFE; Liquid Isolation-free Enrichment), and have validated next-generation sequencing (NGS) and PCR-based technologies on cfDNA enriched directly from plasma/serum, eliminating the requirement for DNA isolation/purification. This compares favorably to current approaches as it avoids significant sample loss, i.e., no extraction no loss, which can greatly affect outcome data, and gives greater confidence in accurate variant calling and quantification of variant allele frequencies.[18-21] The extraction-free wash-free seamless protocol is highly automated, with minimal hand-on time that can process >350 samples in 4 h. Thanks to its outstanding efficiency and yield, all genetic testing, either somatic or germline, DNA or RNA, tissue or liquid biopsy,

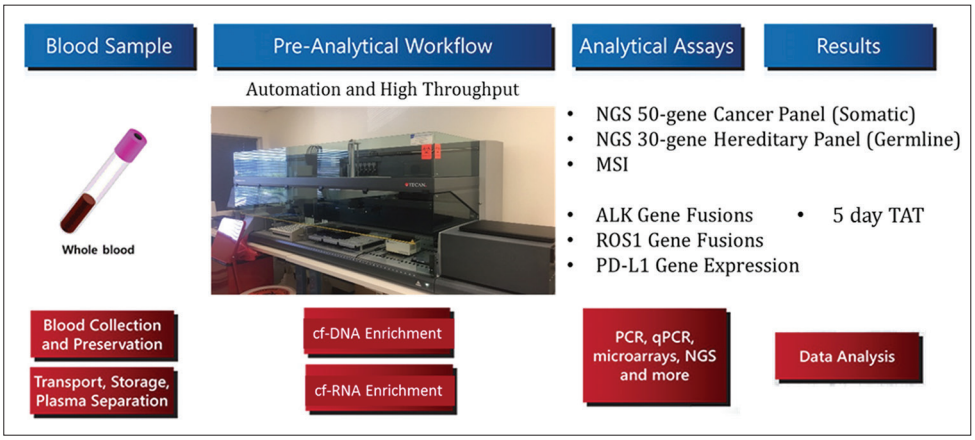

Figure 1: *In situ* cfDNA enrichment workflow deployed in a clinical laboratory for precision medicine. TAT: Turnaround time

Table 1: Summary of *in situ* genetic technologies

Technology	Sample processing	Multiplexing	Automation	Endpoints	Applications
In situ PGR	Yes, require tissue sectioning	Possible but difficult	No	Qualitative measurement of "cellular" DNA/RNA	Detection of target gene/transcript
In situ hybridization	Yes, require tissue sectioning	Limited by fluorophores	Partially	Quantitative measurement of "cellular" DNA/RNA	Detection of target chromosome/gene/ transcript
In situ sequencing	Yes, require tissue sectioning	Yes	No	Transcriptome-wide quantitative measurement of "cellular" RNA	Genome-wide profiling of gene expression *in situ*
In situ direct-on-specimen enrichment	None	Yes	Yes	Quantitative measurement of "cell-free" DNA/ RNA; Cell-free nucleic acid preparation	Liquid biopsy genomic analysis

Table 2: Comparison of *in situ* genetic technologies

Technology	Key steps	Phase in workflow	Year of invention
In situ PCR	Tissue preparation; cell permeabilization; *in situ* amplification; detection	Analytical detection	Early 1990s
In situ hybridization	Probe preparation; Pre-treatrnent of tissue; hybridization; detection	Analytical detection	1969
In situ sequencing	Tissue fixation/permeabilization; Rolling-circle amplification; Sequencing; detection	Analytical detection	2003
In situ direct-on- specimen enrichment	Biofluid specimens; extraction-free wash-free *in situ* enrichment; library prep; sequencing	Pre-analytical sample preparation	2015

RCR: Polymerase chain reaction

can be performed with just one tube of blood (2–4 mL). In contrast, other laboratories require multiple tubes of blood (>20 mL) to get enough of cfDNA for one single NGS analysis [Figure 1].

CONCLUSION

This review presents a perspective of *in situ* genetic technology, including a summary of the methods, principles, endpoints, and applications [Table 1]. These procedures are used to target, detect, and quantify the genetic material at cellular level. The class of *in situ* genetic techniques has been used for direct measurement of gene functions in specific cell population without nucleic acid isolation. The endpoints for most *in situ* techniques are relatively downstream for analytical detection purpose, except for cfDNA *in situ* enrichment which is focused on pre-analytical sample preparation step [Table 2]. The interest in *in situ* enrichment of cfDNA is based on the idea of liquid biopsy, to recover as many circulating cfDNA molecules as possible, thereby reducing sample loss and improving assay sensitivity.

Due to rapid advances in genomic medicine, genetic analyses have become routine in clinical practice and research. During the past decade, landmarks have been made using *in situ* genetic technology to unravel underlying mechanisms of tissue-specific gene expression and regulation. Looking forward, *in situ* genetic methods will become widely accessible and feasible to perform even for small size laboratories, which coincided with developments in gene and/or cell therapy. With the launch of the prototype of *in situ* cfDNA enrichment for personalized medicine, we are at the beginning of an era that will provide new horizons in human health care.

REFERENCES

1. Nuovo GJ. Co-labeling using *in situ* PCR: A review. J Histochem Cytochem 2001;49:1329-39.

2. Bagasra O. Protocols for the *in situ* PCR-amplification and detection of mRNA and DNA sequences. Nat Protoc 2007;2:2782-95.
3. Nuovo GJ. In: Darby IA, editor. *In situ* Localization of PCR-amplified DNA and cDNA. Methods Molecular Biology. Vol. 123. New Jersey: Humana Press; 2000. p. 217-38.
4. Pesquet E, Barbier O, Ranocha P, Jauneau A, Goffner D. Multiple gene detection by *in situ* RT-PCR in isolated plant cells and tissues. Plant J 2004;39:947-59.
5. Dapson RW. Macromolecular changes caused by formalin fixation and antigen retrieval. Biotech Histochem 2007;82:133-40.
6. Madrid MA, Lo RW. Chromogenic *in situ* hybridization (CISH): A novel alternative in screening archival breast cancer tissue samples for HER-2/neu status. Breast Cancer Res 2004;6:R593.
7. Ehtisham M, Wani F, Wani I, Kaur P, Nissar S. Fundamentals of *in situ* hybridization: A review. Int Res J Clin Med 2016;1:23-9.
8. Raff R, Schwanitz G. Fluorescence *in situ* hybridization: General principles and clinical application with special emphasis to interphase diagnostics. Int J Hum Genet 2001;1:65-75.
9. Camidge DR, Kono SA, Flacco A, Tan AC, Doebele RC, Zhou Q, *et al.* Optimizing the detection of lung cancer patients harboring anaplastic lymphoma kinase (ALK) gene rearrangements potentially suitable for ALK inhibitor treatment. Clin Cancer Res 2010;16:5581-90.
10. Mass RD, Press MF, Anderson S, Cobleigh MA, Vogel CL, Dybdal N, *et al.* Evaluation of clinical outcomes according to HER2 detection by fluorescence *in situ* hybridization in women with metastatic breast cancer treated with trastuzumab. Clin Breast Cancer 2005;6:240-6.
11. Speicher MR, Carter NP. The new cytogenetics: Blurring the boundaries with molecular biology. Nat Rev Genet 2005;6:782-92.
12. Diez-Roux G, Banfi S, Sultan M, Geffers L, Anand S, Rozado D, *et al.* A high-resolution anatomical atlas of the transcriptome in the mouse embryo. PLoS Biol 2011;9:e1000582.
13. Battich N, Stoeger T, Pelkmans L. Image-based transcriptomics in thousands of single human cells at single-molecule resolution. Nat Methods 2013;10:1127-33.
14. Raj A, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S. Imaging individual mRNA molecules using multiple singly labeled probes. Nat Methods 2008;5:877-9.
15. Lee JH, Daugharthy ER, Scheiman J, Kalhor R, Yang JL, Ferrante TC, *et al.* Highly multiplexed subcellular RNA sequencing *in situ*. Science 2014;343:1360-3.
16. Lee JH, Daugharthy ER, Scheiman J, Kalhor R, Ferrante TC, Terry R, *et al.* Fluorescent *in situ* sequencing (FISSEQ) of RNA for gene expression profiling in intact cells and tissues. Nat Protoc 2015;10:442-58.
17. McLarty JL, Yeh CH. Circulating cell-free DNA: The blood biopsy in cancer management. MOJ Cell Sci Rep 2015;2:21.
18. Ford A, Brown C, Yeh CH. Sample preparation of circulating cell-free DNA by direct-on-specimen and silica-based methods. J Biol Sci 2017;3:23-35.
19. Spurgin J, Ford A, Athanasuleas J, Yeh CH. Next-generation targeted sequencing of circulating cell-free DNA from droplet volumes of blood. Int J Life Sci Res 2015;3:23-9.
20. Ford A, Brown C, Yeh CH. Pre-analytical assessment of circulating cell-free DNA prepared by an isolation-free enrichment technology. Acta Sci Cancer Biol 2018;2:2-6.
21. Javadi N, Ford A, Yeh CH. Blood-drop liquid biopsy for monitoring mutation load and therapeutic responses. Canc Ther Oncol Int J 2016;1:555572.

How to cite this article: Ford A, Brown C, Caver E, Yeh C. The Evolution of *In Situ* Genetic Technology. Journal of Clinical Research in Oncology 2018;1(1):1-5.