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Microarrays towards nanoarrays and the future Next Generation of Sequencing methodologies (NGS)

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ABSTRACT

This Review analyze insides for Next Generation of Sequencing (NGS), and discuss the state of the art based in different techniques and strategies. In this way it was presented from amplified and modified methodologies towards non-amplified methods for varied genomic materials. Thus, it was discussed and compared the basis of both methodologies. By this manner it was afforded to understand how the specific non-covalent nucleotide interactions where involucrated and participated in the oligonucleotide detections. Therefore, insides of new strategies and approaches by the applications of different strategies such as enhanced targeted signal detections from single oligonucleotides pairs were showed and discussed. In this manner, it was revised and discussed representative developments from the state of the art of nanoarrays, colorimetric methodologies, use of molecular and polymeric transducers, Fluorescence Resonance Energy Transfer (FRET), Metal Enhanced Fluorescence (MEF), Enhanced Plasmonic approaches, non-classical light coupling methods, and use of semiconductors, as well as other strategies. By this manner it was intended to introduce and discuss the future of NGS methodologies from insides and advances of different approaches discussing the already incorporated NGS in the market. In addition, it was afforded to some insights about how this technology it is currently developed and applied for broad spectra of applications within different Research Fields as Omnics.

1. Introduction

In order to afford the discussion of future Next Generation of Sequencing (NGS) [1], it should be understood the most well-known biotechnology development nominated as Polymer Chain Reaction (PCR) [2] as well as new modified related methodologies. These technologies are currently used for different applications from routine genomic analysis towards research studies within broad spectra of interests.

The PCR method consists in a complex system based on enzymatic engineering that could read the targeted DNA and incorporate by nucleophilic substitution complementary oligonucleotides [3]. Thus, from very low oligonucleotide concentrations found in real samples, it could be augmented concentrations to levels that could be detected and quantified by a colorimetric technique. Hence, this is an amplified DNA detection methodology that was expanded and broadly incorporated in the market. However, this methodology it could be considered as more time consuming with costs associated with specific Biological and Chemical reagents. Therefore, it is still being of interest the development of modified methodologies or other derivatives methods based on PCR [4]. For example, looking for minimize volumes and quantities as well time it could be mentioned the developments of confined PCR assays within microfluidics droplets on Chips [5], an alternative and portable DNA amplification technology such as loop-mediated isothermal amplification (LAMP) [6] and recombinase polymerase amplification (RPA) [7]. But, all the mentioned methodologies were based on amplifying the DNA samples to generate measurable concentrations to be detected with advanced and standard instrumentation available in biochemistry laboratories. Moreover, it should be mentioned the robust development of quantitative Polymerase Chain Reactions (qPCR) is used to monitor relative changes in very small amounts [8]. This methodology was based on traditional PCR; but with the incorporation of a reverse transcription of total RNA or mRNA to complementary DNA (cDNA) by the application of the enzyme reverse transcriptase [9];

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followed by a quantification based on the use of varied Fluorescent reporters [10]. By this manner, for example it was recently showed their applicability in an optimized detection of intra- and extra-cellular SARS-CoV-2 RNA [11] For all the mentioned methods the non-covalent interactions between complementary oligonucleotides were the basis of the recognition [12], incorporation of complementary oligonucleotides, and final amplification by iteration of many cycles to obtain larger amounts of genomic material [13]. In addition the fluorescence techniques and methods used for quantifications were as well by specific non-covalent interaction between apolar and conjugated organic molecular structures intercalated within different nucleotide bases [14].

In similar manner, later it was developed the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) [15] biotechnology to repair genomic material or incorporate a new one [16]. This biotechnology is still being developed and discussed for human applications. However, it is clear and well-studied how it works. In brief this relative new technology is based on an complex enzymatic system that permit to act as enzymatic scissors in targeted genes coupled to the incorporation of genomic material replacement [17]. By this manner, it was recently awarded two researchers working at Max Planck Unit for the Science of Pathogens, Germany; and at University of California, USA with the Nobel Prize in chemistry 2020 for “the development of a method for genome editing” [18]. Therefore, using the CRISPR/Cas9 it was developed a methodology to change DNA of animals, plants, and microorganisms with extremely high precision. In addition, looking for new methodologies for DNA detection and sequencing, it should be highlighted that the CRISPR could be adapted to the use for targeted DNA or RNA detections by a proper incorporation of fluorescence labelling in this complex enzymatic system. [19,20,21]

Moreover, it should be mentioned other methodologies in close relation with PCR such as exponential Isothermal Amplification Assays of Nucleic Acids for proteins, cells, small molecules, and enzyme activities [22].

For these amplification assays were eliminated the particular required condition of temperature cycling for PCR. In similar manner, other developments should be mentioned as strand displacement amplification (SDA), rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP), nucleic acid sequence based amplification (NASBA), helicase-dependent amplification (HDA), and recombinase polymerase amplification (RPA).

In all these methods should be highlighted that the amplification should be defined as an amplification of the quantity of the genomic material with a consequent improved detection for quantification. In addition, other approaches and strategies were proposed and contemplated to increase the signal from low concentrations within real samples with ideally minimal sample manipulation. In this direction it should be mentioned strategies contemplating the incorporation of fluorescence, colorimetric, chemiluminescence, Raman spectroscopy, and electrochemical approaches that enabled to higher sensitive detections of varied new approaches of study. However, Remaining issues, such as undesirable background amplification resulting from nonspecific template interactions were discussed and opened further studies to improve other techniques.

About other technologies well know developed that it should be mentioned the northern blotting as variation of the southern blotting. This technique was used for detection of RNA instead of DNA. The cellular RNAs should be extracted from varied tissues; and fractionated according to size by gel electrophoresis. And as for southern blotting, the RNAs are transferred to a filter and detected by hybridization with a radioactive probe. In this manner, it was began studies of gen expressions by RNA detection within different type of cells [23]. In similar manner, other labellers were used such as fluorescent reporters as it was used for the technique Fluorescence In Situ Hybridization (FISH). By this manner it was achieved to the hybridization of human chromosomes with chromosome-specific fluorescent probes that label each of the 24 chromosomes a different color [24]. And as well it was used antibodies, and labelled antibodies for DNA and RNA labelling and complementary strands targeting [25].

However, the detection signals recorded are still being considered an issue to improve and enhance, and challenge to overcome. Therefore, there is a particular need of research and development related with the transduction and amplification of the signal in the place to amplify quantities. This concept generated the possibility to afford studies on single cells, or reduced quantities of real samples; where later the known Next Generation of Sequencing afforded these types of insides and today it is possible to propose other levels of research studies. Within these new approaches it should be highlighted the developments based on tools made of light by incorporating varied molecular reporters that could provide signal transductions from nuclei acids interactions.

As an important approach to these techniques it should be mentioned and highlighted the real-time DNA Sequencing from Single Polymerase Molecules [26]. In this approach it was performed a real time sequencing from DNA polymerase for uninterrumped template directed synthesis using four distinguishable fluorescently labelled deoxiribonucleosides triphosphates (dNTPs). By this manner it was detected the enzymatic incorporation of single oligonucleotides through a single DNA strand on a zero-mode waveguide nanostructure arrays (ZMW). The conjugation of the terminal phosphates of dNTPs permitted the tracking in real time of the DNA synthesis (Fig. 1). At this level, the labelling, the non-covalent interactions, and detection of nucleotides is closely related within levels of Single Molecule Detections (SMD) [27].

In addition, it should be mentioned the high impact by extension of genomics research towards OMNICS applications by tracking of DNA and RNA expressions. In this manner it was developed other approaches to detect a broad spectrum of biomolecules generated from genomic translations with direct implication of varied illness. For example, it could be mentioned the whole-genome mapping of small-molecule targets for cancer medicine [28]. For this research study it was stimulated the genomic expression by varied drug administrated. Thus, it was tracked the transcriptome level using Next-Generation Sequencing (NGS) technology; where it was not amplified quantities of genomic material, but enhanced signaling permitted the detection of low variations in the genomic material constitution in presence of targeted small molecules.

As well it was developed single-cell RNA and DNA sequencing technology that avoided multi-steps methodologies based on improved detection efficiencies of low natural genomic material concentrations within cells [29]. Thus, it could be highlighted how from the control of single molecule detection, nucleotides, single and double stranded DNA or RNA it was afforded to studies within single Biostructure levels. Furthermore, developments of nanomedicine such as by sampling biological fluids to study glioblastoma pathogenesis were permitted just only with NGS methodologies due to the low concentrations of genomic material tracked within extracellular vesicles in blood samples, from circulating tumor cells, and circulating tumor DNA. By this manner, it was discussed about current treatments not so effective and other approaches such as immunotherapy. [30]

In summary, along this introduction it was briefly presented a large and broad theme for the detection of variable oligonucleotide sequences. Thus, it was discussed current methodologies such as PCR, Western blott, etc. and related techniques with the application labelling strategies. These methodologies were classified as amplified techniques due to it were increased quantities of the genomic material from real samples. In addition, it was introduced other methods with the incorporation of varied labelling techniques. These techniques joined with the accurate non-covalent nucleic acids interactions permitted tracking from single genomic bases to oligonucleotides. Moreover, it was showed how important were the enzymatic bioassays in the amplification methods as well new ones developed. In this manner, other approaches could be proposed and discussed such as light responsive nanobiotechnology based on improved signals transductions, cascade signaling, enhanced signals, etc.; and new combined methods by

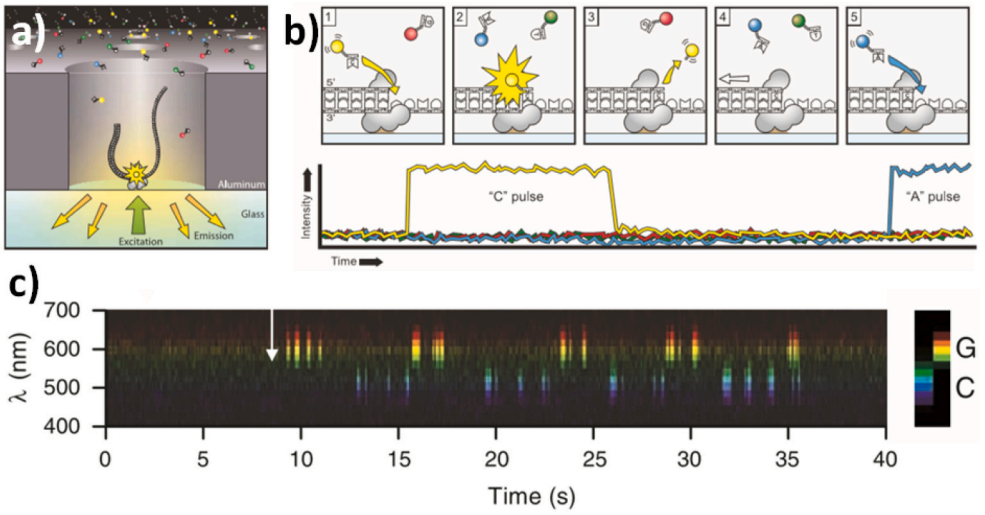

Fig. 1. Principle of single-molecule, real-time DNA sequencing. a) Experimental geometry. a) single molecule of DNA template-bound F29 DNA polymerase is immobilized at the bottom of a ZMW, illuminated from below by laser light. The ZMW nanostructure provides excitation confinement in the zeptoliter (10^{-21} l) regime, enabling detection of individual phosphor-linked nucleotide substrates against the bulk solution background as they are incorporated into the DNA strand by the polymerase. b) Schematic event sequence of the phosphor-linked dNTP incorporation cycle, with a corresponding expected time trace of detected fluorescence intensity from the ZMW. (1) A phosphor-linked nucleotide forms a cognate association with the template in the polymerase active site, (2) causing an elevation of the fluorescence output on the corresponding color channel. (3) Phosphodiester bond formation liberates the dye-linker-pyrophosphate product, which diffuses out of the ZMW, thus ending the fluorescence pulse. (4) The polymerase translocates to the next position, and (5) the next cognate nucleotide binds the active site beginning the subsequent pulse. c) Time-resolved fluorescence intensity spectrum from a ZMW. Data from a 15 × 5 pixel area from each movie frame were spatially collapsed to a 15-pixel spectrum, shown as a function of time. The arrow denotes addition of the catalytic metal ion that initiated the polymerization reaction. Reprinted with permission from S. Turner et al. Copyright 2009 Science (cite [29]).

incorporating enzymatic methods. Some of the mentioned were partially developed and reported; however many of them were not developed as best of our knowledge. Therefore, it was developed new technology such as from nanoarrays to the Next Generation of Sequencing (NGS) already incorporated in the market as well as within a broad research field nominated as Omnics.

In this perspective, in this short communication it was reviewed recent developments with high impact within NGS techniques and methods. By this manner, it was afforded different strategies to collect targeted signaling from single nucleotides, oligonucleotides, RNA molecules, and single and double DNA chains, towards new sequencing methodologies. Thus, it was highlighted non-amplified methods; and focused on varied and accurate controlled chemical and physical phenomena to lead to improved and enhanced methods.

2. From Nobel prizes in chemistry to basis of DNA micro-arrays technology

From the Nobel Prize in chemistry 1980 related with sequencing nucleic acids of different Biostructures such as bacteria [31], towards PCR [32,33] and CRISPR [34] awarded with Nobel Prize in chemistry 2020 [35], for genome detection and edition respectively; it could be mentioned some of the fundamental variables involucrated. So, the most important variables for success in the targeted application involucrated could be nominated as: i) the interaction of nucleic acids; and ii) the implication of biostructures such as bacteria and enzymes that permitted the biosynthesis and edition of genomic material. Thus, in brief, all the components for the genomic edition and detection should be accurately placed in confined structures. These biosystems based on the non-covalent and covalent bonding afforded to specific bioconjugated oligonucleotides. By this manner, it was detected amplified genomic material within intermediate to high concentrated intervals of concentrations.

The amplified techniques are currently used with acceptable bioanalytical performances such as accuracy, precision, and reproducibility; however it is considered as high time consuming methodologies with a high cost related with the use of specific reagents and quantities required.

In this context, it should be highlighted the development of nano-arrays for targeted oligonucleotide detection. Thus, from the concept and approach towards real applications were discussed in this section. In addition, it was introduced to the NGS.

The knowledge and development within genomics and synthesis of oligonucleotide libraries joined chemical modifications of the material genetic afforded to the targeted interactions with complementary DNA strands of variable lengths. This was the beginning of the basis of new approaches of detections. This proof of concept was later grafted on different structures such as array and nanoarrays. By this manner, a new technology was developed with particular challenges within different research fields involucrated for these fabrications. In this perspective, it should be mentioned as for example, the advanced development and easy access of genomic material on the market that still permitting the design of new experiments in different research fields as well as within nanotechnology for genomics. As a representative example of the concept and potential applications within life-sciences it could be mentioned the chemical modification of glass with combinatory chemical methods to obtain high density synthetic oligonucleotide arrays [36]. The arrays have been designed and used for quantitative and highly parallel measurements of gene expression, to discover polymorphic loci and to detect the presence of thousands of alternative alleles of cellular analysis.

From this high technology beginning it was leaded to a large number of variables to control, improve, and develop new approaches. About these main variables it could be highlighted: i) the signal detection with the complementary DNA target recognition; and ii) faster and low cost methodologies. In this way, the signal tracking based on imaging techniques permitted to incorporate developments of spectroscopics, photonics, and nanophotonics, with variable mode of energies. In all the mentioned research fields the improved sensitivity, precision and accuracy of the oligonucleotide or DNA strand detection was the key factor. Thus, the addition of classical light and non-classical light was, and still being, an important part of the array detection system. For example, it could be mentioned the light-directed synthesis of high-density oligonucleotide arrays using semiconductor photoresists [37]. These micro-meter sized modified surfaces permitted the targeted fluorescent labelled detection of complementary targeted DNA strands recorded by fluorescence microscopy. By this manner it was showed the accurate mapping of fluorescence hot spots just only in the right

complementary modified sites. Therefore, it was combined a high sensitive spectroscopical technique that permitted the imaging of DNA detection. These types of research developments opened to the interest of increased imaging resolution with detection of lower DNA concentrations [38].

From the beginning of the development of this technology, it was afforded the discussion about its implications and future within life sciences. So, it was leaded to potential impact on the human health by the accurate acquisition of genomic material for precision medicine applications. And at this level the use of high density microarrays to hybridize target DNA strands were evaluated to identify individual bases modifications. [39] So, it was presented in advance the challenges and goals for the next generation of sequencing methodologies. Thus, the human genome project was based on different methodologies with varied sequencing techniques. However, faster techniques were always desired for DNA sequencing. In this perspective, in the beginning with high time consuming methods developed by the pioneers in this field, such as Gilbert and Maxam [40], it was later developed by Sanger et al. several faster, more efficient techniques to sequence [41]. But, even further developments, was achieved by a company of automation. This automated method was based on the Sanger et al. technique, involving a purified DNA polymerase enzyme to synthesize DNA chains of varying lengths; coupled to a capillary tube electrophoresis for their separation with a single nucleotide resolution [42]. It should be noted that this method used dideoxynucleotide triphosphates (ddNTPs). These chain-terminating dideoxynucleotides lack the 3′ hydroxyl (OH) group needed to form the phosphodiester bond between one nucleotide and the next during DNA strand elongation. Thus, when a dideoxynucleotide is incorporated into the growing strand, it inhibits further strand extension.

In this manner, nanoarrays was funded based on strong bases in the beginning accompanied with implications of high impact such as the human genome project. Within the different technologies applied it should be highlighted the DNA microarrays; that contained unless 1000 strands with variable nucleic acids covalent bonded to a micro-meter active surface. Thus, it was used to the detection of the relative concentrations of varied nucleic acids sequences by hybridization and posterior detection of the targeted and natural hybridization event. The detection could be achieved as for example by a proper fluorescence labelling (Fig. 2) [43].

Therefore, it was designed 2D and 3D bottom ups with not only covalent bonding, as well it was used non-covalent linking. However, it should be mentioned that even if this technology was automatized with the incorporation of micro-systems, devices and robots, it was replaced slowly by the other DNA sequencing technologies. But, the micro-arrays afforded to gene studies such expressions, transcription binding analysis [44], and genotyping [45].

It should be mentioned that these DNA arrays applied showed some limitations related with; i) the generation of DNA libraries; ii) non-lineal signaling with consequent quantification issues; iii) aggregation problems; iv) specificity; v) signal recorded, and vi) non-detection of particular sequences non grafted on surfaces [43]. Each of these mentioned items are still being challenges, and many of them were improved by new approaches of array fabrications.

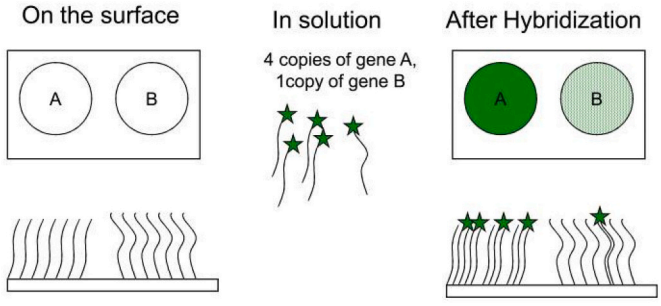

Fig. 2. Simplified view of a DNA array. The upper rectangles show two spots of DNA on a solid surface (sequences "A" and "B") prior to and after hybridization. The lower rectangles show highly idealized side views of the same surfaces. Reprinted with permission from R. Bumgarner et al. Copyright 2013 Curr Protoc Mol Biol. (cite [43]).

However, the incorporation of sensitive signaling of fluorescence as it was showed previously permitted to the detection of specific hot spots with varied colours joined with artificial intelligence analysis. This afforded to Single Nucleotide Polymorphisms as well as Multi-Nucleotide Polymorphisms detections within short time of intervals. In this context, the use of microarrays showed excellent performances joined with analysis hybridization data. These experiments consisted in 32 autosomal and eight X-linked genomic regions, each consisting of ~50 kb of unique sequence spanning a 100-kb region, in 40 humans [46]. By this manner, each human genome site was scanned the fluorescence intensity by dividing each feature into 56 equal-sized pixels. Thus, it was obtained the fluorescence intensity from 30 pixels from the raw data measured by the detector. This analysis was highlighted by the capability to record information of single pixel analysis (Fig. 3).

As it was showed in this example by combining targeted human DNA libraries, with high sensitive signaling, analyzed by image data collection with single resolution pixel, afforded to detection of complementary DNA strands. Therefore, with perspective of improvements should be developed other Optical approaches and strategies to overcome varied problems depending of the targeted application. In this perspective, in the last years many approaches and applications were reported.

3. Recent developments of micro-, and nanoarrays for genotyping and sequencing

As it was discussed previously the use of arrays based on chemical surface modification joining genomic material such as aptamers, oligonucleotide, RNA and DNA chains of variable lengths with controlled and known sequences with a single nucleotide resolution permitted the detection of complementary strands. In these approaches, were combined two key elements; first the quantity of genomic material to be detected, and secondly the generation of the library of the biological sample to be studied as well as the applied genomic array to decode the

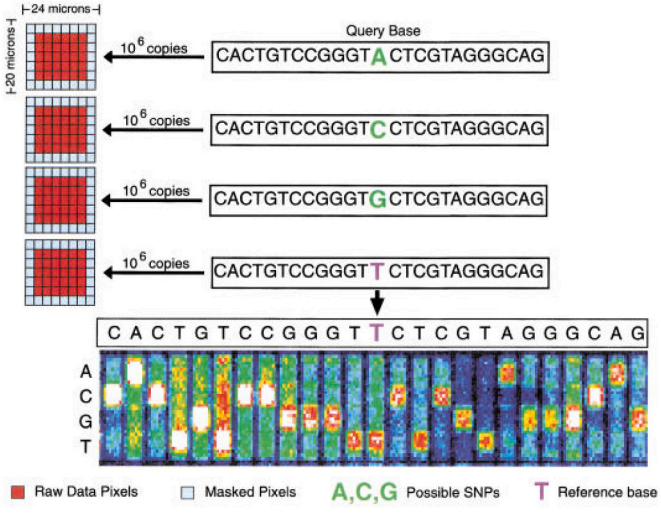

Fig. 3. Eight features (four for the forward strand and four for the reverse complement strand) are associated with every queried site. Each feature consists of a 25-base oligonucleotide. The 13th base is the query base and all possible genotypes are tested. Each feature is divided into 56 equal pixels, and the pixels are scanned individually. The outermost 26 pixels are "masked," so that only the 30 interior pixels are used for any calculation. Reprinted with permission from D. J. Cutler et al. Copyright 2001 Genome Res. (cite [44]).

targeted sequences. Within all these themes, the subject accessibility and costs associated was contemplated on Single Nucleotide Polymorphisms (SNP) microarrays [47]. In this perspective microarrays technology showed lower costs than other new technologies available in the market. Thus, high-throughput DNA microarrays from early 2000s showed powerful platform for simultaneously analyzing hundreds of thousands of SNPs, evaluating copy number variant (CNVs) in single experiments, and densities of single nucleotide variants related with specific sequences of genes too [48].

In this manner it was studied and elucidated varied challenges within genotyping for varied targeted interests in this field. This was permitted by the combination of a huge big data available of genes that permitted the detection of targeted sequences; and the customization of arrays depending of needs. In addition, it should be highlighted that the whole genome data sequences was joined to artificial intelligence data analysis for studies of targeted genomic lines in different real samples. And, as it was mentioned previously the quantity available was and currently is an important parameter to control; which it is still being mainly overcame by coupling PCR amplification to microarrays applications.

Therefore, in the last years it was increased this research field accompanied with a large number of products on the market. So, implications of available arrays on the market, customized arrays on demand, collaborative Research works with institutions and companies capable to manage genomic materials and their modifications, and potential new approaches were reported. All of them, and other ones non-mentioned could be involucrated in these technologies as well as other approaches are applied for targeted applications within genomics with particular emphasis in genotyping due to the biological implications, impact on bio-informatics, precision medicine, and new emerging research field in the cutting edge of knowledge within life sciences.

By this manner, for example it could be mentioned analysis of genomic variability of vegetables, such as new SNP genotyping technology for Target SNP-sequencing and its application in genetic analysis of cucumber varieties [49] In this research work was developed a new methodology exploiting the multiplex PCR amplification and microarrays with higher through sequencing compared to KASP [50], GBS [51], and other SNP sequencing methodologies [52]. This improved method was made by a library generation of 261 cucumber varieties for targeting SNP-seq with 163 perfect SNPs from 46,12,350 SNPs based on 182 cucumber re-sequencing datasets. By this manner it generated a microarray with a higher capability to detect genomic variability within different cucumber origins from Asia and Europe. In this methodology it should be highlighted the use of imaging based data analysis generated by a matrix derived from differential SNP genotypes detected (Fig. 4).

Moreover, it should be highlighted advanced developments that arrived to products on the market by the main and most important high technological companies involucrated in sequencing technologies. In this way, it could be mentioned rapid low-cost microarrays-based genotyping for genetic screening in Primary immunodeficiency [53]. This DNA array detection system was proposed as the Next Sequencing Array technology due to their large versatility of uses with minimal quantities of genomic material required. At the best the genome-wide Illumina Global Screening Array (GSA) was developed to perform with 200 ng of DNA. So, the quantities required were minimal with low cost involucrated. By this manner, it was analyzed immunodeficiency disorders (PIDs) in humans. Thus, Probes were custom-designed to genotype 9415 variants of 277 PID-related genes. And the validation of genotype calling was performed by comparing the GSA with whole-genome sequencing (WGS) data of 56 non-PID controls. In addition, DNA samples of 95 clinically diagnosed PID patients, of which 60 patients (63%) had a genetically established diagnosis PID panels or Sanger sequencing, were analyzed to test the performance of the GSA (by NGS). And, additional SNVs detected by GSA were validated by Sanger sequencing. So, multi-Omnics approaches were applied to confirm an accuracy rate of 99.7%,

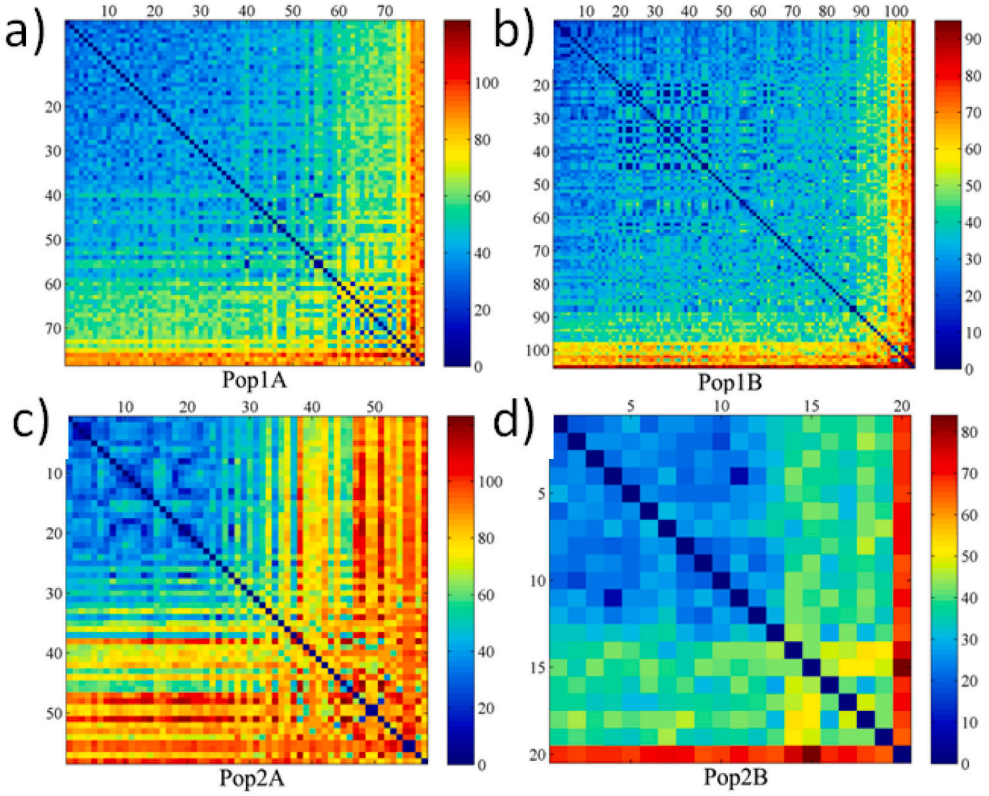

Fig. 4. Heatmap of pairwise comparison matrix derived from differential SNP genotypes in Pop1A (a), Pop1B (b), Pop2A (c), and Pop2B (d). Blue to red indicate the increasing number of differential SNP genotypes. Reprinted with permission from L. Zhang, et al. Copyright 2020 Sci Rep (cite [49]). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

accompanied with a sensitivity for detecting rare PID variants as high of 87%. Finally, it should be highlighted the importance and involucration in these technologies of artificial data analysis. In this example it was used a software provided by the company nominated as Illumina GenomeStudio 2.0, Biodiscovery Nexus 10.0, and R-3.4.4 software, that permitted a faster analysis and interpretation of the data recorded.

In addition to genotyping immunology applications for humans, it could be added the high interest to detect resistant pathogens against antibiotics in order to apply precision medicine approaches. As for example the case of genotyping of methicillin resistant staphylococcus aureus [54]. In addition it could be mentioned other examples related with other objectives such as the exploration of the gene evolution within varied interval of times. In this perspective, recently it was reported insights into the loss of the Y chromosome with age in control individuals and in patients with age-related macular degeneration using genotyping microarray data [55].

Finally, it should be mentioned that this type of arrays due to its accessible library making, with resolution at single nucleotide level, accompanied with low cost associated, permitted to be extended animal models research as well. For example, it was recently reported the characterization of a haplotype-reference panel for genotyping by low-pass sequencing in Swiss large white pigs [56]. In this work, it was analyzed the genetic diversity between two lines of Swiss large white pig breed. By this manner it was possible to detect considerable differentiation of two split populations that occurred within just only few generations ago (Fig. 5). Moreover, it should be highlighted that summed to the low cost it was increased the variant density more than 350 folds.

Therefore, it could be noted valuable information recorded in this study such as for example the following characteristics. It was estimated runs of homozygosity (ROH) character for 19,146, 365 biallelic Single-nucleotide polymorphism (SNP) to investigate genomic inbreeding in the two lines. Thus, from a total 111,201 ROH, it was determined an average length of 391.28 kb (ranging from 50 kb to 11.1 Mb). The ROH contained an average number of 3176 SNP. The boars from the dam and sire line had 1604 ± 133 and 1575 ± 91 ROH with an average size of 377,928 and 402,731 bp, respectively (Fig. 5). The genomic inbreeding (FROH, i.e., the fraction of the autosomal genome covered by ROH), was 0.26 ± 0.03 and 0.28 ± 0.03 for the dam and sire line, respectively. Thus, it was classified the ROH into short (50–100 kb), medium (100 kb - 2 Mb) and long ROH (above 2 Mb). However, most ROH belonged to the medium length class. The average FROH was similar in both lines for small and medium ROH. But it was highlighted that, FROH was higher for long ROH in the sire line.

In addition, as concluding remarks from an overview and evaluation of different genotyping arrays [57], it could be highlighted variable performances depending of the targeted applications. in this perspectives varied arrays and methodologies were used for clinical applications such as gen activations, organelle genomics, mithocondrial DNA (mtDNA), pharmacogenetic genes depending of the actual status of encoding genes related with pharmacophores interactions or required treatments, HLA genes (human leukocyte antigen), analysis of Structural Variant (SV) or CNV, and density of single polymorphisms. This is just an important point to take into account due to differences in the different real samples as well as the versatility offered from the main companies related. Moreover, other new approaches such as miniaturized on beads arrays Chips should be considered for SNP Discovery [58] with low cost, effective accompanied with high throughput characteristics. So, the possibilities have been increased drastically in the last years that should be known too. In similar manner, it should be highlighted the degree of precision and accuracy in high sensitive diagnosis as for example to predict genome-wide abnormalities in embryos [59] with consequent future perspectives within gene therapies and precision medicine. However, there were still being extended the concept of arrays a well towards the nano-scale. In this context, it could be mentioned the apparition of nano-arrays based on varied nanomaterials and strategies.

Moreover, after varied genotyping arrays developments it was introduced from the control of nano-scale towards responsive surface generation for targeted DNA chains. This incorporation opened the management of other sizes that afforded to new optical approaches such as the use of Chips [60], and modified beads [61]. For example, not so far ago it was reported the complete human genome sequencing using unchained base reads on self-assembling DNA nanoarrays [62]. This was a huge research work where many researchers from academia and company were joined to develop a genome sequencing nanoplatform with efficient imaging performances and low consumable costs. It was applied a combinatorial probe anchor ligation chemistry to independently assay from patterned nano-arrays of self-assembling DNA nanoballs with high nucleotide resolution. By this manner, three human genomes afforded to an average of 45- to 87-fold coverage per genome and identifying 3.2 to 4.5 million sequence variants per genome. These results, showed a validation sequence accuracy of about 1 false variant per 100 kilobases. And it was highlighted the low cost associated with few thousands of dollars for this complex analysis.

However, these research interest was not closed. At the place it was still being reported many other research studies in relation of other approaches such as further studies with massive parallel sequencing on

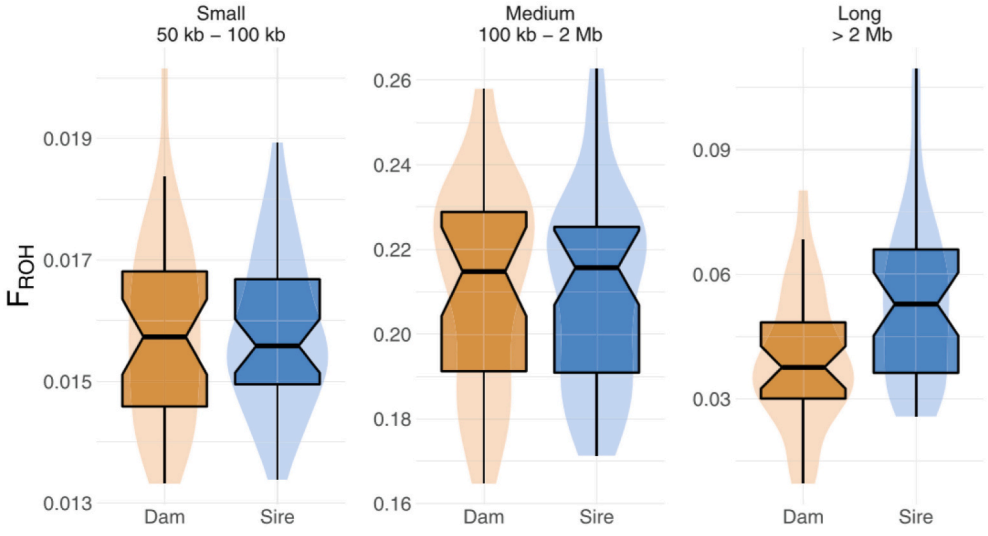

Fig. 5. Genomic inbreeding in the two lines. FROH in dam and sire line, estimated for three groups of ROH classified based on their length: small (50 kb – 100 kb), medium (100 kb – 2 Mb) and long (>2 Mb). Reprinted with permission from N.K. Kadri et al. Copyright 2021 BMC Genomics, Springer (cite [56]).

DNA nano-arrays [63]. In this approach was coupled a free-PCR Nano-Balls array of 200 nm sizes each that afforded by rolling circle replication accompanied with three folds times of improved signaling and elimination of free dye linkers nominated as scar interferences. These performances were afforded by incorporation of dye labelled reversible terminated nucleotides that permitted DNA imaging detection.

In addition, other nano-approaches and emissions modes were developed as well. Gold nanoparticles accurately modified with varied partial complementary DNA aptamers afforded to targeted single DNA chains detections. This approach was based on the nano-aggregation of the DNA grafted gold Nanoparticles in presence just only of the complementary DNA chains. Thus, it was coupled Enhanced Plasmonics (EP) properties to Luminol Chemiluminescence for the generation of Enhanced signaling [64].

Moreover, it should be mentioned Enhanced coupled phenomena such as Fluorescence Resonance Energy Transfer (FRET) and Metal Enhanced Fluorescence (MEF) for DNA detection. This proof of concept was first developed by the deposition of non-covalent interaction of labelled double stranded DNA with a polymeric transducer on MEF silver Core-shell Nanoplatforms. By this manner, the emission of the adsorbed fluorescent triplex was absorbed and re-emitted by an acceptor Fluorescent Silica shell placed at an optimized distance of the silver core for MEF. Thus, it was recorded higher emissions from both coupled phenomena [65]. Then, with the perspective of label-free DNA Biosensing, it was optimized the Nanoarchitecture based on multilayer fluorescent nanocomposite using the previous polymeric transducer mentioned [66]. In this research work, it was optimized sizes of silver cores, spacer lengths, donor/acceptor spectroscopical properties complementarity, covalent bioconjugation of DNA probes, and the optical set-up used for detection. For this study, it was developed an In Flow optical set up for nano-imaging detection and tracking of targeted single complementary DNA strands. Therefore, it was applied for direct molecular detection of SRY gene from unamplified genomic DNA by MEF-FRET coupling [67]. So, further studies were developed to applying this nanoplatform and optical set up for PCR-free blood group genotyping [68].

In this manner, it could be highlighted the importance of the nanoscale control and properties generated from within the nanomaterial towards the external part and functionalization in its close surrounding. This particular need joined with high smart responsive materials as it was discussed previously could afford to the proposal of Single Molecule Detection (SMD) approaches. For example, it could mentioned the recent publication related with bench-top fabrication of Single-Molecule Nanoarrays by DNA origami placement [69]. In this approach it was developed modified grids which combined the design of micro-arrays and the capability of SMD by DNA origamies and top down litography to place these bio-nano-architectures in patterns with 5 nm of resolution. In this manner it was obtained 100 nm self-assemblies as functional nanodevices. Therefore the control of the nanoscale accompanied with the generation of nano-patterns leaded to produce smart molecular responsive nanoplatforms deposed on modified substrates that could provide innovative nano-arrays and microarrays for genotyping applications. And with these perspectives, it still being of high interest the design of Plasmonics platforms by engineering nano-sphere arrays with gold-DNA core–shells and junctions as opto-responsive sensors [70]. So, gold nanoparticles could provide with controlled fluorescence emissions potential smart responsive ultraluminiscent gold core-shell nanoplatforms for genotyping applications based on MEF nano-Imaging [71]., within In-Flow approaches [72,73] and modified substrates [74].

In addition to plasmonics and coupled phenomena, it could be highlighted in similar manner the FRET as high sensitive and accurate enhanced non-classical light [75] for targeted detection of biomolecules and biostructures [76] with potential use for genomic material detection. For example, recently it was reported FRET Bio-assays on soft Nanoballs for specific and amplified detection of microRNAs [77]. Moreover, other approaches such as massively parallel sequencing using antibodies specific to each natural nucleobase [78] showed new innovative ways to afford towards labelling of genomic materials with potential use in genotyping. In this way, other innovative approaches were proposed for the well-known NGS for bioinformatics analysis such as family genetic inheritance [79]; and prospective assessment of the clinical benefit of a tailored cancer genes and targeted therapies applied for recurrent or metastatic head and neck cancer [80]. These examples were just only to mention some of the uses of fundamental research for targeted applications within genotyping. However, other new technologies as for example towards the Next Generation of label-free devices for health monitoring were presented based on porous silicon-based aptasensors [81]. In this manner, it was reported advanced label free optical approaches on modified porous silicon that acted as transducer surfaces for the detection of pathogenic microorganisms and diagnostic molecules with high sensitivity, and low limit of detections (LOD).

In this manner, it could be highlighted in this section the importance of the control and functionalization of varied sizes of substrates with targeted DNA libraries from micro-, towards nano-scales and incorporated as well in smart responsive surfaces. Thus, devices with different responses could be achieved. This is the general concept of material modifications applied for micro-, and nano-arrays developments with varied types of signal transducer after an accurate recognition of complementary DNA strands. Therefore, it was introduced the versatility of the nano-scale tuning within the NGS technology. So, in the next section it was afforded to present many uses and applications from fundamental to applied research by NGS technology.

4. State of the art in Next Generation of Sequencing (NGS)

In this section it was intended to introduce NGS from the market to the research bench. By this manner, it was leaded to an overview of basis and capabilities of the available technology. Thus, it could be mentioned that basically the genotyping could be developed in three steps: i) cluster generation, ii) sequencing by copying genomic strands, and iii) data analysis. [82] Moreover, it should be contemplated that in many cases the genomic material should be extracted and by this manner depending of the real sample it could be available different techniques and kits for i) the extraction, to then ii) generate the genomic Library, and iii) quantify; to finally apply variable softwares and artificial intelligent data analysis. Thus, it is possible to classify the recording in different manners such as by the use of Library Barcoding [83]. In this context, it should be noted that the basis of the sequencing is based on the awarded P. Berg, W. Gilbert, and F. Sanger with the Nobel Prize in Chemistry in 1980 [84]. So, it should be noted that Gilbert has studied these parts of bacteria chromosome which control the reading or transcription of the genetic information; while Sanger developed a method to determine the sequence of human DNA. These strong bases permitted then to copy and quantify oligonucleotides based on modified methodologies. By this manner it was achieved the NGS technology with the capability to read, copy and quantify genomic content of single cells. Thus, by applying basically these three steps mentioned, it is involucrated the genomic material fragmentation, the addition of adapters or labellers depending of the methodology used, to then sequencing, copy and reassembling the different fragments to form a genomic sequence. While the detection it could be in a similar way as capillary electrophoresis. But, the critical difference is that NGS sequences millions of fragments in a massively parallel fashion, improving speed and accuracy while reducing the cost of sequencing. In addition the whole methodology was optimized to low quantities of real sample and all the procedures could even being automatized [85]. As for example, one of the last single-molecule sequencing systems have been drastically reduced in size. Oxford Nanopore Technologies' systems such as GridION, MinION or Flongle are portable handheld system for RNA and DNA sequencing for reads of more than 2 Mb. Their detections were developed based on changes in electrical conductivities from DNA strands passing through biological nanopores. Thus, it was resolved varied nucleotide sequences [86].

However, it should be noted that other approaches were developed such as by fluorescent labelling of reversible terminators [87] that were imaged on deoxynucleoside triphosphates (DNTP), to then cleave them to allow incorporation of the next base. Since all 4 reversible terminator-bound dNTPs should be present during each sequencing cycle. This technique and methodology developed permitted an accurate whole Human Genome Sequencing using the mentioned Reversible Terminator Chemistry [88]. In addition, in flow methods could lead to manage low genomic concentrations from single cell analysis by the right coupling of Nanotechnology such as it was included in the Single Cell barcoding (Fig. 6) [89].

In this manner, it should be highlighted that different NGS technologies are currently ongoing within varied research studies with varied interest. Therefore, it was observed from recent reported research works a large diversity of targeted studies accompanied with improvements and accurate advices related with what is the best option to use depending of needs. Hence, there is a huge support researcher could propose and advance new insides in different topics within genomics. As for example, it could be noted recent important developments using different NGS technology associated with different interests. In this perspectives it should be highlighted the RNA sequencing for transcriptomic studies [90]; where the quantification was required and afforded by parallel RNA sequencing [91]. By this way it was analyzed transcriptome profiling of activated T cells [92].In similar direction, it should be highlighted the use of NGS for DNA sequencing [93] looking for high impact research studies such as targeted deep resequencing of the human cancer genome [94]. Thus, it was achieved improved diagnosis and precise therapy application of lung cancer patients [95]. And, these analyzes showed as sensitive to detect somatic mutations in cerebral cortical malformations [96]. So, a large number of variables that could affect human genome as well as to other living organisms could be studied. In similar manner, this technology could be applied for precision medicine research and applications.

5. Perspectives in NGS

In the previous sections, it could be highlighted the importance of the basis of the recognition events joined to varied strategies for sequencing. This was leaded by detections from Single nucleotides towards oligonucleotides and higher strand lengths. Therefore, it was discussed about micro-arrays, nano-arrays for genotyping based on different strategies. Moreover, it was briefly introduced the main and most well-known NGS technologies used in varied genotyping research studies; leading to the critical steps involucrated and challenges afforded successfully. From all the mentioned themes and topics, it was noted the importance of the recognition and library generation, time consumed, automatization; and even in some cases costs. In addition, it should be added the special need of improvements in the targeted detection signals from perfect nucleotide matching to single nucleotide-, as well as for multiple- nucleotide mismatching.

In this direction, it could be noted how it was transferred knowledge from fundamental research to applied studies and products on the market within this research field. For example, it could be mentioned how it was transferred nanotechnology with nano-arrays towards DNA detection, accompanied with genomic engineering. Moreover, it could be noted other example from development of controlled nano-porous materials combined to well-known and applied techniques such electrophoresis, that leaded to one type of NGS placed on the market. While, the incorporation of fluorescent labellers joined to a bio-machinery afforded to multiple-coloured detection arrays. Thus, it is important to track the state of the art in different Research field that could contribute to new approaches for genotyping. In this perspective enhanced, and amplified signals based in different strategies are highly desired. And from this point of view; Enhanced signaling is related with the development of an increased signal based on coupled single physical and chemical phenomenon to varied coupling phenomena; while amplified the augmented signal is related with laser bases developments.

Therefore, variable sensitivities should be managed in order to detect varied lengths and nucleobases constitutions from DNA strands. Thus, it could be mentioned many approaches recently reported in scientific literature based on different strategies. For example by absorption techniques, it was developed the amplification-free colorimetric test for sensitive DNA detection based on the capturing of gold nanoparticle clusters [97]. There, it was amplified the DNA detection based on the combination of on the capture of multiple gold nanoparticle clusters onto the surface of magnetic microbeads, leading by this manner to an increase of the plasmonic signal and, thus, of the overall sensitivity. Moreover, the use of new fluorescent DNA labellers, it was afforded to single molecule polarization microscopy of DNA intercalators that leaded to accurate detailed conformational structure of S-DNA. (Fig. 7)

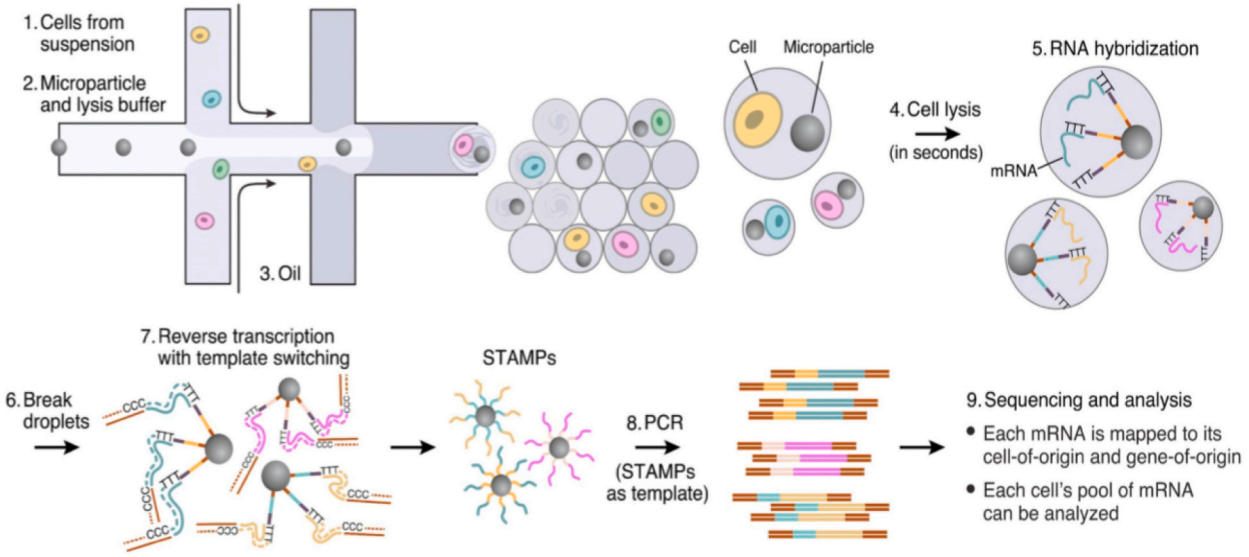

Fig. 6. Single-cell barcoding. Automated microfluidics solutions can provide additional features for library preparation. Liquid droplets containing cell, Microparticles with adapters and reagents are generated in an immiscible oil phase. Each droplet contains a specific bar code,which allows a separable library preparation for every single cell. Reprinted with permission from M. Goldman et al. Copyright 2015 Cell (cite [89]).

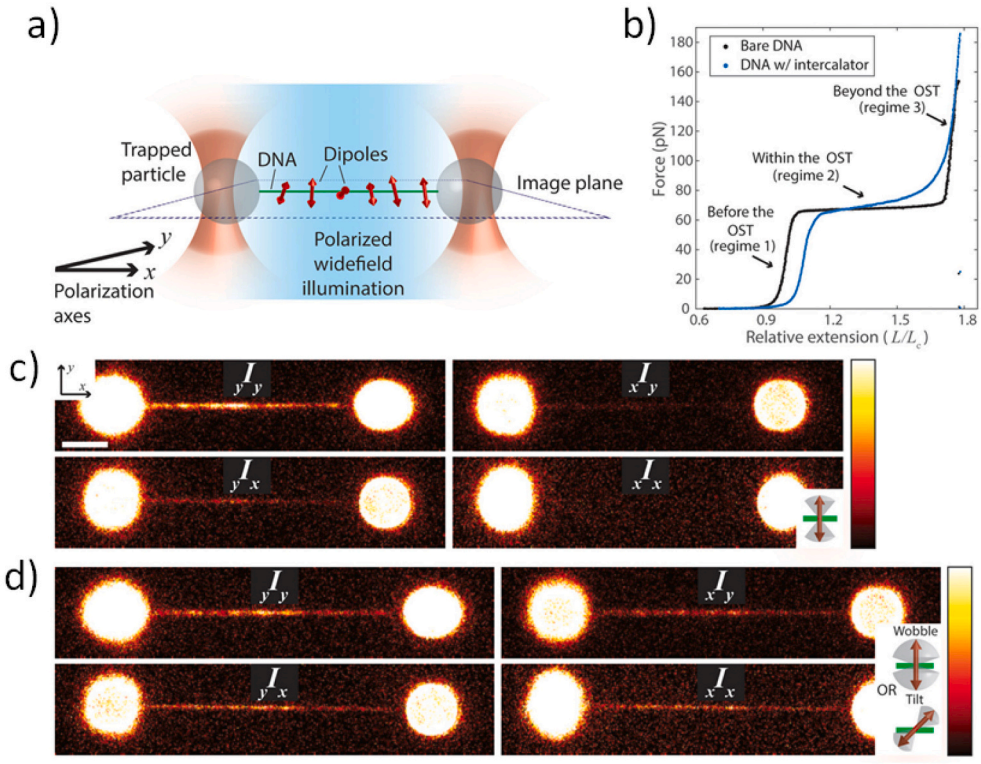

Fig. 7. Intercalated DNA fluorescence changes polarization after the OST. a) Optical tweezers align and stretch a DNA molecule within the microscope image (x/y) plane. Polarized laser light excites intercalated dyes, and corresponding fluorescence is resolved into x- or y-polarized components using a polarizing beamsplitter. Dipoles of intercalated dyes are depicted as bidirectional arrows. b) Typical force-extension curves for both bare DNA and DNA in the presence of ~10 nM YOYO-1. While the two curves differ slightly, in both cases, three distinct regimes can be distinguished: before the OST (regime 1), within the OST (regime 2), and beyond the OST (regime 3). c) and d) Fluorescence polarization images show DNA densely coated with YOYO-1, extended horizontally to regimes 2 and 3 [(c) and (d), respectively] (scale bar, 5 μm). Insets illustrate the possible intercalator dipole orientations (red) with respect to the DNA axis (green) that could induce the observed fluorescence polarization features. Gray cones depict wobble regions from which dipoles may depart from their mean orientations. Note that in the increased wobble hypothesis [upper inset of (d)], dipoles explore a greater range of orientations due to a wider wobble cone. Reprinted with permission from E. J. G. Peterman et al. Copyright 2019 Sci. Adv. (cite [98]). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[98].

However, for improved signaling, specificity, and information required from single nucleobases constitutions afforded to new assays based on other coupled phenomena. Thus, Fluorescence Resonance Energy Transference (FRET) [99] was applied as well. This phenomenon was used in different approaches such by DNA origamies for sensing applications based on FRET [100]. In this study it was showed the accurate effect of distances between donor and acceptor dyes from different sizes achieved in Nano-arrays.

Moreover, it should be mentioned ultra-luminescent properties are based on a plasmonic effect named Metal Enhanced Fluorescence (MEF). Metal enhanced fluorescence (MEF) is a plasmonic effect that enhances the emission fluorescence of a substrate placed at a given distance from a metallic surface [101]. The MEF effect depends of the distance of the fluorophore from the metallic surface due to the electromagnetic field intensity decays exponentially ($1/r^3$) affecting by this manner drastically the fluorophore excitation [102]. For this reason in order to evaluate this parameter there are many studies developed using polymeric spacers as silica. In these Nanoarchitectures, the fluorophore is covalent bonded and the concentration can be controlled for maximal enhancements. These studies are in progress over surfaces [103] and colloidal dispersions [104], depending of the nanoarchitecture design and applications.

Moreover, MEF-FRET coupling phenomena could be even more sensitive accompanied with higher enhancements. In this manner, it was developed Plasmon-Enhanced Resonance Energy Transfer from a conjugated polymer to fluorescent multilayer Core−Shell nanoparticles [105]. In this research work it was showed and improved coupling phenomena with strong plasmonics properties from silver Cores. The fluorescent polymer emission could be intercalated within DNA strands covalently attached on silver Nanoparticles; and by this manner recognize complementary DNA strands. The triplex forms of intercalated fluorescent polymer within complementary DNA strands increased its emission due to a modification from planar to anti-planar conformation accompanied with a major rigidity in comparison with the duplex form. In this manner, it was afforded to a label-free biosensor based on multilayer fluorescent nanocomposites and a cationic fluorescent polymeric transducer (Fig. 8) [106].

Thus, each hybridization event was signaled by a potentially large number of excited reporters following the efficient plasmon-enhanced energy transfer between target-activated polymer transducer and fluorophores located in the self-assembled core−shell aggregates. It was achieved direct molecular detection of target nucleic acids at femtomolar concentrations. This approach and proof of concept was applied for the direct molecular detection of SRY gene from unamplified genomic DNA by metal-enhanced fluorescence and FRET [107] And proposed as PCR-free blood group genotyping [108]. In addition it should be mentioned that these mentioned achievements were developed within In Flow systems coupled to imaging detection. In this perspective recently it was reported about dynamics of phenomena of real-time monitoring of bead-based DNA hybridization in a microfluidic system; where it was inferred about amplicon hybridization behavior on solid supports and dynamics phenomena related [109]. Moreover it should be highlighted that in this case it was applied a modified cartridge substrate joined to a pneumatic module that afforded to the detection of Single Nucleotide Polymorphisms.

Finally, other materials associated with different properties could be applied such as from semiconductive nanoparticles, nano-transducers and neural networks on Chips based on DNA-nanoparticles. In these organized systems varied matter properties interacting are potentials centers for DNA detections and further applications. In this perspective it was recently reported nanoparticle-based computing architecture for nanoparticle neural networks where it was decoded information from single molecular level and genomic material incorporation (Fig. 9)

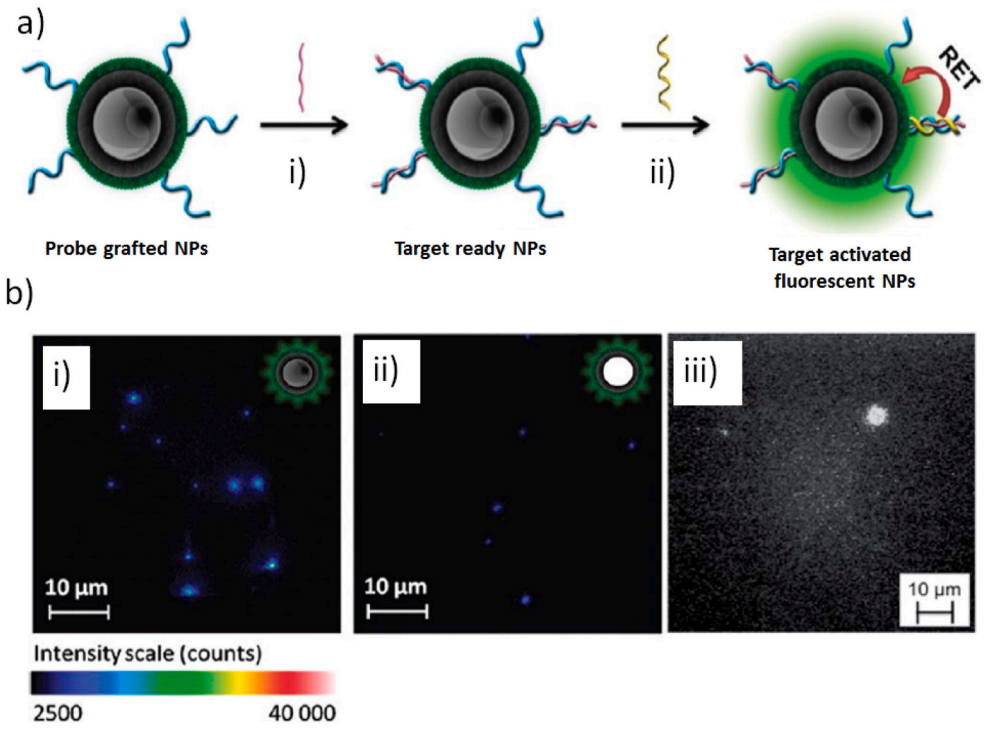

Fig. 8. Principle of DNA detection on fluorescent multi-layer core-shell NPs: a).i) target ready NPs are prepared by complexing ssDNA probe grafted NPs with polymer transducer; ii) hybridization of target DNA with ssDNA probes activates the polymer transducer as energy donor towards dye doped silica shell and excitation at 410 nm generates fluorescence emission by acceptor molecules at 550 nm. Reprinted with permission from D. Boudreau et al. Copyright 2011 ACS Nano (cite [99]). b) i) and ii) effect of the Core on the NPs detection event in presence and absence of the silver template respectively; iii) The 3 s detection limit for perfectly matched targets is 1×10^5 molecules per mL, or 0.2 fM. Right: example of a fluorescence image captured by imaging flow cytometry. Reprinted with permission from D. Boudreau et al. Copyright 2013 Analytical Methods (cite [106]).

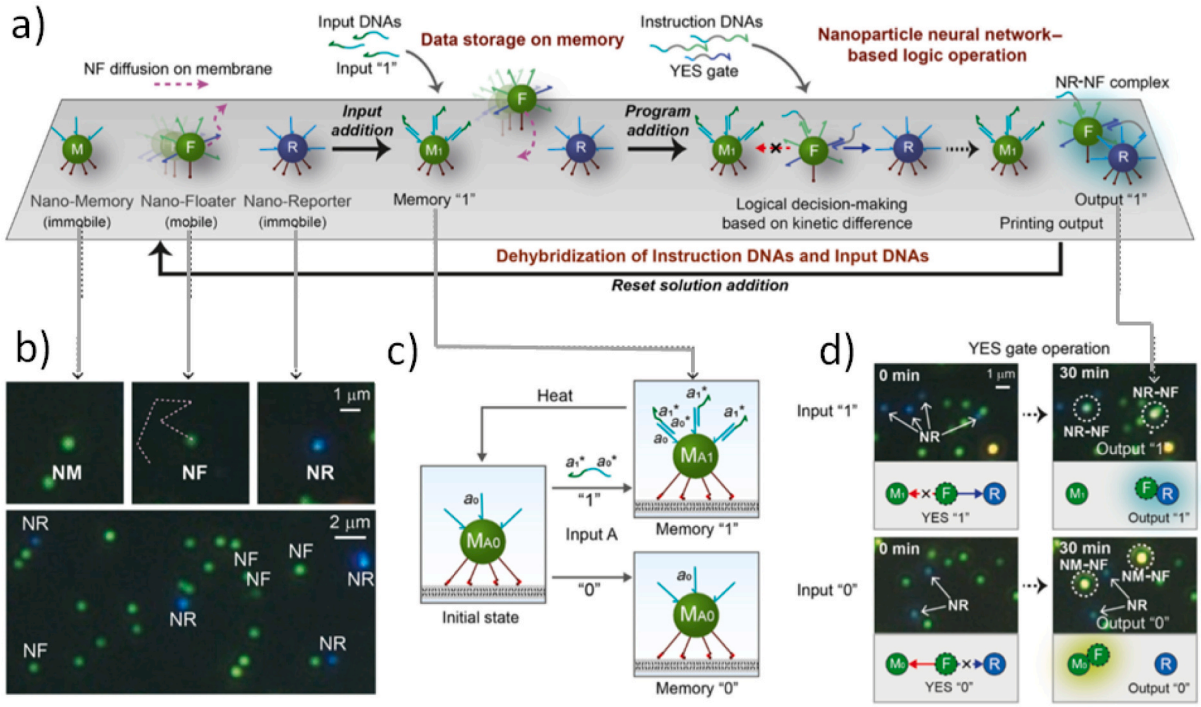

Fig. 9. a) NVNA on an LNT chip (i) data storage on NM, (ii) neural network (NNN) operation by Instruction DNA set addition, and (iii) reset by dehybridizing DNAs for the next executions. b) Time-lapse dark-field microscopic imaging can differentiate each nanoparticle on LNT via scattering color and mobility. The nonlabeled nanoparticles are NM. c) Molecular information storage on the NM changes the exposed single-stranded domain. d) YES gate operation results. Input "1" results in output "1," printing the NF-NR. Otherwise, all NFs are trapped to NM and exhibit no reaction on NR, which is output "0." Note: NVNA corresponds to Nanoparticle-based VNA from Von Neumann architecture (VNA). And LNT chip as deposed lipid bilayer on substrate or Lipid NanoTablet. Reprinted with permission from J.-Min Nam et al. Copyright 2020 Sci. Adv. (cite [110]).

[110].

In this direction it should be added the incorporation of nano-emitters on variable modified substrates to transduce signals through the material as well as to detect single molecular events. Thus, nano-platforms with the capability to detect single DNA strands or to generate nano-, and micro-arrays are desired from varied points of view due to

their potential uses within devices fabrications with targeted functions.

Therefore, by an accurate control of varied modes of energy and signaling could be leaded to DNA detection with improved performances that would be welcomed within the future NGS. In this direction, it could be possible to incorporate nanomaterials with enhanced performances for the design of new smart responsive materials. Maybe, it sounds easy, however it is not. And it is opened to all the interested in this high impact research field. So, all these studies and related are of high interest and impact within genomics and potential future of NGS.

6. Conclusions

In this communication was introduced to the highlights and basis taken into account for the development of DNA detection systems with influence in the actual NGS technology from the early beginning with the Nobel Prize in chemistry 1980 related with sequencing nucleic acids from bacteria. Moreover, its relation with the largely expanded and applied PCR technology actually in use. As well as the CRISPR technology awarded with the Nobel Prize in chemistry 2020 for the development of a method for genome editing. In this manner, for all the mentioned developments accompanied with applied technology it was involucrated fundamental variables such as nucleic acids recognitions incorporated within Biological systems such as, bacteria and enzymes. Thus, natural and synthetic catalytic approaches focused on confined spaces permitted to place complementary nucleotides to create more DNA strand copies. Then, in the next step different methods of detections permitted its quantification. In this perspective in all the different approaches developed were taken into account both main and crucial steps.

By this manner, micro-arrays towards nano-arrays to iterate and propose new alternative methodologies for genotyping was considered both mentioned parameters. And it should be highlighted that the improved performances were from the control of the molecular level and complex bio-machinery that permitted to place in the right positions single nucleotides to be coupled in optimal conditions of reactions. In this context, it was contemplated the synthesis of DNA libraries and the application of varied detection techniques. In this context, it should be mentioned as important factor to control and develop for success, the high sensitivity required of the detection technique applied. In particular from real samples; where the genomic material concentration is low, and requires amplification of its quantity. So, Micro-arrays and PCR technologies were developed and actually still being widely used in research studies as well as in clinical analysis. In this context, for micro-arrays it was highlighted both mentioned vital variables to afford to significative quantities of genomic material coupled with optical detection techniques such as colorimetric and fluorescence signaling. In addition, it should be noted the importance of the optical set ups used as well as the targeted source of photons from single nucleotides. In this aspect, the nano-arrays from the developments of nano-optics it was fundamental for advances in this research field. Thus, it should be differentiated the early beginning of nano-arrays that it is well accepted, available, and well placed on the market; with the upcoming new nano-optical arrays. Therefore, it was discussed about other physical phenomena that could be exploited controlling chemistry and optics; such as enhanced absorption, fluorescence, energy or electronic conductions, resonant energy transfer; and coupled phenomena. And, with this perspective as well it could be mentioned the signal amplification based on the concept of laser mechanism where the signal is augmented due to a high sensitive detection signaling and increased transduction nominated as amplified within laser research field.

By this manner, it was discussed based on recent developments by Colorimetric methods, FRET, MEF, MEF-FRET coupling, and Enhanced semi-conductions based on the design of targeted nano-optical platforms. Therefore it was afforded to the discussion of possible incorporations and new developments of the future NGS. Moreover, in this entire context it should not be forgotten the important factor that whole new system ideally should be automatized. Thus, inflow methods, imaging, signal Waveguiding [111], micro-devices and nano-devices [112] could afford to the future of this technology [113,114].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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