Top Med Chem (2020) 33: 523–560
DOI: 10.1007/7355_2019_82

Published online: 28 November 2019

Advanced Assays in Epigenetics

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Contents

Abstract Epigenetic mechanisms orchestrate the finely tuned regulation of genetic material and play a pivotal role in defining cellular functions and phenotypes. A growing set of tools supports analysis of the epigenome. This chapter will provide an overview of the principle methods of studying complex epigenetic machinery, focusing on recent advancements of tools and techniques in the field of epigenetics. It will also address the advantages, limitations and perspectives of each approach. Increasingly, the high sensitivity, specificity, accuracy, precision and reproducibility of cutting-edge technologies in epigenetics are allowing the identification of new key targets and molecular mechanisms in healthy and pathological states and are becoming methods of choice for clinical investigations.

Keywords Epigenetics, Genome, Histone modification, Methylation, miRNA

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Abbreviations

5caC	5-Carboxylcytosine
5fC	5-Formylcytosine
5hmC	5-Hydroxymethylcytosine
5mC	5-Methylcytosine
AlphaScreen	Amplified Luminescent Proximity Homogeneous Assay Screen
BRET	Bioluminescence resonance energy transfer
BS-seq	Bisulfite sequencing
CAB-seq	Chemical modification-assisted bisulfite sequencing
CE-SSCP	Capillary electrophoresis single-strand conformation polymorphism
CETSA	Cellular thermal shift assay
ChIP	Chromatin immunoprecipitation
ChroP	Chromatin proteomics
ddPCR	Droplet digital PCR
EDC	1-Ethyl-3(3-dimethylaminoproyl)-carbodiimide hydrochloride
EnIGMA	Enzyme-assisted identification of genome modification assay
ePL	Enhanced ProLabel
ES	Embryonic stem
EWAS	Epigenome-wide association studies
EXPAR	Exponential amplification reaction
fCAB-seq	5-Formylcytosine chemical modification-assisted bisulfite sequencing
FISH	Fluorescent in situ hybridization
FLIM	Fluorescence lifetime microscopy
FRET	Förster resonance energy transfer
G4	G-quadruplex
HATs	Histone acetyltransferases
HMTs	Histone methyltransferases
HTDR	High-throughput dose-response
HTS	High-throughput screening
HT-seq	High-throughput sequencing
ISH	In situ hybridization
ITC	Isothermal titration colorimetry
LC-MS	Liquid chromatography-mass spectrometry
LNA	Locked nucleic acid
miRNA	microRNA
miR-TRAP	miRNA trapping
MPS	Massive parallel sequencing
MS	Mass spectrometry
MST	Microscale thermophoresis
NGS	Next-generation sequencing
Nluc	NanoLuc luciferase

PAR-CLIP	Photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation
QD	Quantum dot
RBPs	RNA binding proteins
RIME	Rapid immunoprecipitation mass spectrometry of endogenous protein
Rluc	Renilla luciferase
RRBS	Reduced representation bisulfite sequencing
scBS-seq	Single-cell bisulfite sequencing
scM&T-seq	Single-cell genome-wide methylome and transcriptome sequencing
scRRBS	Single-cell reduced representation bisulfite sequencing
snmC-seq	Single-nucleus methylcytosine sequencing
SNPs	Single-nucleotide polymorphisms
SPR	Surface plasmon resonance
TAB-seq	Tet-assisted bisulfite sequencing
TCL	Targeted chromatin ligation
Tm	Melting temperature
TR-FRET	Time-resolved fluorescent energy transfer
UV	Ultraviolet
YFP	Yellow fluorescent protein

Epigenetic modifications work with genetic mechanisms to control gene expression and chromatin structure in normal cells [1]. Disruption of epigenetic regulatory processes results in abnormal gene function and/or alterations in cellular signalling pathways, leading to several pathological states such as cancer [2]. Unlike most genetic mutations, epigenetic abnormalities are reversible and potentially good targets for deriving therapeutic strategies. Global changes in the epigenetic landscape can precede genetic alterations at early stages of disease development, making them suitable predictive biomarkers for clinical diagnosis [3]. Furthermore, several studies have identified defects in epigenetic modulators as indicators of both progression and outcome of a disease, as well as biomarkers of patient response to therapy and early detection of recurrence, suggesting a prospective translational application in clinical practice to improve prognosis [4–7]. Advanced technologies able to rapidly analyse epigenetic changes have revealed a challenging scenario and provide an accurate detection of new key epi-targets in pathological states and several multifactorial complex diseases, including cancer. This chapter will describe the most innovative epigenomic technologies and will be divided into three main sections: (1) assays, in vitro and *in cell*, useful to define the activity and binding of epigenetic modulators; (2) genome-wide approaches used to determine the chromatin modification status of cells at nucleotide resolution level and to detect the binding sites of DNA-associated proteins; and (3) methodologies for miRNA expression and functional analysis. The advantages, limitations, cost-effectiveness and therapeutic perspectives of each technique will also be discussed. Further work improving the

sensitivity, specificity and reproducibility of epigenetic technologies will not only reveal underlying deregulated epigenetic pathways associated with disease progression and treatment response but will also provide a robust measure of novel biomarkers for risk stratification in terms of disease development and for individualized precision medicine.

1 Epi-proteomic Analyses

Post-translational histone modifications make up a significant portion of epigenetic mechanisms involved in several biological processes. Chromatin remodelling is known to affect up- and downregulation of gene expression, and tumorigenic diseases can be driven by alterations of epigenetic modification patterns. New technologies have therefore been designed to identify histone modification enrichment in specific cellular contexts, and new drugs able to restore the physiological epi-state have been developed. This section of the chapter focuses on the techniques most commonly used to rapidly identify macromolecular complexes and to discover drugs that bind and modify protein activity. The assays have been divided into two main groups: (1) in vitro assays following protein expression and purification and (2) techniques that allow experiments to be performed directly in cells.

1.1 Biochemical Studies: In Vitro Assay

The choice of technique mainly depends on the starting point and objectives. The advantage of biochemical experiments is that the protein of interest can be isolated, purified and overexpressed. Data analysis is straightforward and fast. Indeed, this approach is commonly used to detect protein-protein or protein-drug interactions in high-throughput screening (HTS) strategies. Cell-based assays allow investigation of the target protein, and all the macromolecular complexes of which it is a part, under physiological conditions. In this case it is important to take into account several factors that can complicate studies in cells, such as drug permeability and half-life, cellular enzymatic degradation, small amounts of starting material and long lead times of experiments. The analysis of chemiluminescent protein interactions (AlphaScreen/AlphaLISA), variation in diffraction radius (surface plasmon resonance), or changes in molecular solvation (isothermal titration calorimetry) are the most common techniques used for in vitro macromolecule interaction studies.

1.1.1 AlphaScreen/AlphaLISA

Biomolecular interactions, post-translational modifications or activity of substrates as competitor compounds can be investigated using AlphaScreen and AlphaLISA

(PerkinElmer) technologies (where Alpha stands for Amplified Luminescent Proximity Homogeneous Assay Screen). These are non-enzymatic in vitro assays that detect protein interactions using donor and acceptor beads. When the interaction takes place, a chemical reaction produces an amplified signal. After donor excitation, singlet state oxygen molecules react with acceptor fluorophores, if located in proximity, that emit light at 520–620 nm [8]. Generally, donor beads in AlphaScreen and AlphaLISA contain a phthalocyanine molecule that produces a singlet state oxygen, keeping energy irradiation at 680 nm. Acceptor beads contain thioxene, anthracene and rubene in AlphaScreen and thioxene, europium, terbium or samarium in AlphaLISA [9]. The beads are covered with reactive aldehydes to bind the ligand, antibody or substrate [9]. Compared to other methods such as time-resolved fluorescent energy transfer (TR-FRET), these assays have better sensitivity and can be used for large-scale reactions. Depending on the interaction under investigation, the beads are functionalized in different ways. AlphaLISA epigenetic toolbox (PerkinElmer) is used to detect histone methylation and acetylation marks. Using S-adenosylmethionine or acetyl CoA as a cofactor, epigenetic enzymes such as histone methyltransferases (HMTs) or histone acetyltransferases (HATs) react on the specific biotinylated histone substrate. Antibody AlphaLISA acceptor beads and streptavidin Alpha donor beads are then added to measure methylation and acetylation levels. The donor binds the histone substrate by biotin-streptavidin binding and the acceptor captures the modification. If the enzyme works, the acceptor and donor beads are in proximity, and, after laser irradiation, a chemiluminescent signal at 615 nm is generated [10]. The same methodology was used to develop an HTS platform to identify new G9a [9] and JMJD2A [11] modulator drugs.

AlphaScreen and AlphaLISA offer several advantages:

- The difference between donor and acceptor wavelength reduces interference.
- Interactions are detected from sub-nM to μM range.
- The assay is suitable for use in HTS and adapts to 96-, 384- and 1,536-well formats.
- Many biological interactions can be studied.

1.1.2 Surface Plasmon Resonance (SPR)

To study in real time the association and dissociation constants of protein complexes or drug enzymes, SPR is one of the most widely used in vitro assays. The technique measures refractive index changes when there is a variation in mass on a gold chip. Incident light (Fig. 1) strikes electrons at the surface of the metal sensor and converts them into surface plasmon waves, generating an SPR angle. Interaction between the target immobilized on the chip and the captured analyte induces a perturbation at the gold surface and therefore a change in angle of reflection, which can be measured. To improve the optical quantitative detection method, SPR microscopy or imaging (SPRi) technology is used for high-throughput probing of biomolecular interactions. SPRi Biochips™ and SPRi Slides™ are customized for HORIBA SPR imaging

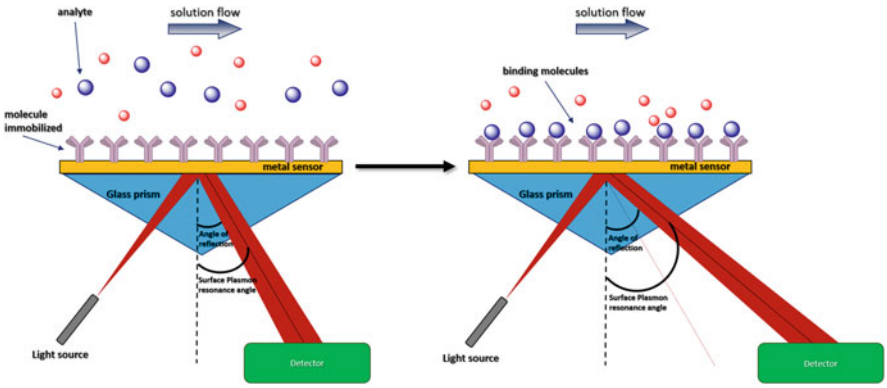

Fig. 1 Scheme of SPR experiment. The molecule is immobilized on metal surface and is injected the analyte solution. When there is the binding between molecules, there is a variation in angle of reflection and of surface plasmon resonance

Table 1 Advantages and disadvantages of SPR

Advantages	Disadvantages
Real-time analysis	Requires a long time for sample preparation
Association (k_a) and dissociation (k_b) constant study	Protein instability
Use of several proteins	It is possible to immobilize only one protein on the gold chip
Does not interfere with physical and chemical molecular properties (e.g. fluorescence of compounds)	
Possible to analyse total protein extraction	
Higher sensibility	

instruments [12]. Although many assays are available to study biomolecular interactions, most require labelled reporter molecules such as fluorophores or antibodies (AlphaScreen, immunoprecipitation [13]) or large quantities of material. The advantage of SPR lies in its reliable instrumentation, automation, disposable sensor chips and versatility of surface chemistries. Table 1 lists the main advantages and disadvantages of the SPR methodology.

1.1.3 Isothermal Titration Colorimetry (ITC)

ITC is a physical technique that allows in vitro study of the thermodynamic parameters of macromolecules or drug interactions in solution. Using temperature variation, it is possible to quantify the interaction by measuring the heat that is released or adsorbed during binding [14, 15]. The microcalorimeter has two cells,

one for water (the control) and one for the sample. The sensor detects temperature difference between the cells. The ligand, loaded by a syringe in a cell sample, binds the molecule, and the heat change is measured compared to the control.

This technique has several advantages: (1) the protein can be left in solution and no molecule modification is therefore required, and it measures the affinity of proteins in their native state; (2) the addition of chemofluorescent tags is not necessary; (3) stoichiometry, association constant and binding enthalpy can be characterized in a single experiment; and (4) experiments can be performed in systems such as dispersions, intact organelles or cells. The main disadvantage of ITC is the limited range for measuring binding affinities (association constants from 10^4 to 10^{-1} M). In addition, it is a slow technique for HTS analysis (0.25–2 h/assay). In recent years, new technologies for a more rapid analysis of data have been designed. In 2017, Mitter et al. described the development of a new ITC technique allowing for the acquisition of data in the order of 0.2 s and less.

ITC is widely used for:

- Quantification of binding affinity
- Confirmation of binding targets of small molecules and characterization of mechanisms of action
- Validation of IC50 and EC50
- Measurement of enzyme kinetics

ITC can be performed using MicroCal™ (Malvern Panalytical) ITC instruments such as MicroCal PEAQ-ITC, which gives high sensitivity and quality data with low sample consumption, or MicroCal VP-ITC, an easy-to-use isothermal titration calorimeter. ITC is mainly used for target validation, measuring enzyme kinetics, investigating the binding mechanisms and potency of drugs and studying the design of inhibitors.

1.1.4 Microscale Thermophoresis (MST)

MST, similarly to ITC, quantifies protein binding in a temperature gradient, but the movement of the molecule and conformational state is recorded by the use of fluorophores [16, 17] (Fig. 2). Infrared laser is used for temperature increase. The molecule is placed in a capillary containing a fluorescent solution, and an infrared ray of a wavelength of 1,480 nm is projected against the capillary, causing an increase in temperature. The fluorophores with an increased temperature are excited and emit fluorescence. The ability to emit fluorescence by temperature increase is inversely proportional to the ability to bind the ligand. MST analysis has no limitations in terms of molecule size or molecular weight and is performed in a matter of minutes in free solution.

There are a number of different devices to monitor MST, such as the Monolith NT.Automated or Monolith NT115Pico (NanoTemper) instruments designed to detect even low picomolar concentrations in microlitre volumes of red-emitting fluorophores. With minimal sample consumption, they provide high sensitivity

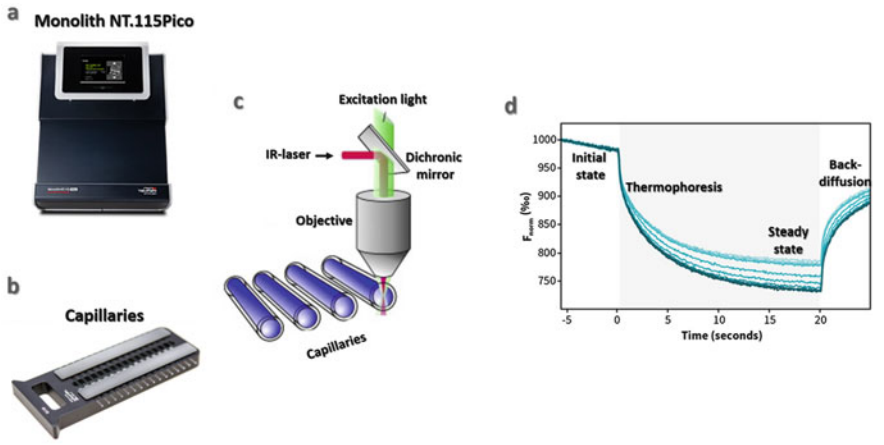

Fig. 2 (**a**) Monolith NT.115Pico. (**b**) Capillary tray. (**c**) Schematic of assay: the temperature is increased inside the capillaries, where the sample is located, by IR-laser. Florescence is detected by the objective. (**d**) Signal obtained in the assay. Molecules in the initial state are homogeneous. When the temperature increases, a T-jump occurs, corresponding to a change in fluorophore properties. The laser is then deactivated and the molecules move by mass diffusion

and affinity interactions. The Monolith NT.Automated is specifically designed with high-throughput capabilities for HTS approaches. In recent years MTS has also been used to study interactions directly in cell lysates using fluorescence fusion proteins. To bypass fluorescence artefacts, MST experiments can be performed with two different cell lysates expressing different fluorescence fusion proteins. These approaches can be used to rapidly test whether and in what manner proteins of interest interact in close-to-native conditions without protein purification. As reported in several studies, MST can be used to characterize protein-histone interactions. Alpatov et al. identified the fragile X mental retardation protein [18] as a chromatin-binding protein that uses its tandem Tudor domain for binding. Josling et al. also demonstrated that the bromodomain protein PfBDP1 binds specifically to H3K9Ac and H3K14Ac peptides with Kd = 110.79 ± 10.31 μM (R2 = 0.995) and Kd = 126.17 ± 14.39 μM (R2 = 0.993), respectively. In conclusion, MST technology is able to perform screening assays, binding assays and competition assays with multiple binding partners [19]. The advantages and disadvantages of these last two techniques are summarized in Table 2.

1.2 Cell-Based Assays

The presence of numerous binding sites, molecular dynamics, interaction complexity and external factors complicates the study of macromolecular complexes in cells. Several new techniques have been developed to study protein-protein interaction

Table 2 Advantages and disadvantages of ITC and MST

Advantages	Disadvantages
• Possibility to measure in one experiment all binding parameters	• Required higher amount of ligand
• It's not required molecular modification to study the interaction	• It's possible to measure only the μM concentrations
• Nothing ligand immobilization	• Kinetics of association and dissociation aren't measured
	• It's a slow technique not used for HTS mode
	• Doesn't read non-covalent complex
• Read small-molecules, vesicles and synthetic compounds	• Required molecular modification with fluorescent tag that can be able to induce a non-specific binding
• Nothing ligand immobilization	• Don't do information on kinetic interaction
• It's possible to measure nM concentrations	
• Faster technique that can be used for HST mode	

complexes, epigenetic modifications and activity of new drugs in living cells. In addition to chromatin immunoprecipitation (ChIP), which has always been one of the most widely used techniques, several methods have been designed to better and more rapidly investigate proteomic complexes, such as cellular thermal shift assay (CETSA) and Förster resonance energy transfer (FRET)/bioluminescence resonance energy transfer (BRET).

1.2.1 ChIP Assay

Immunoprecipitation and ChIP assays are the two main techniques for studying epigenetic modifications and their quantization (ChIP-PCR). The two approaches have also been combined with next-generation sequencing (NGS) to generate profiles of genetic modifications [20] (ChIP-seq), as will be amply described in Sect. 2.1. Over the years, proteomics studies have evolved, coupling conventional ChIP technology that involves cross-linking with formaldehyde, sonication and subsequent quantitative analysis using real-time PCR with mass spectrometry (MS). The combination of these two techniques led to the development of new protocols such as ChIP-MS [21], chromatin proteomics (ChroP) or rapid immunoprecipitation mass spectrometry of endogenous protein (RIME) [22], which allow a faster analysis of all proteins. Standard ChIP assay has several disadvantages, including the possible alteration of chromatin-protein interactions during the experiment, the large amounts of starting material necessary to obtain good results and the long time required to complete the procedure (3 days). A new microfluidic technique was developed to decrease reaction volumes and reduce experiment times [23]. This new microfluidic device integrates sonication and immunoprecipitation, using 1,500–2,000 cells to point (compared to 10^{6-7} in conventional ChIP). Specifically, the cell/DNA

sonication step is integrated with the rest of the procedures, and acoustic energy is used to facilitate the mixing and washing of beads. In this way the experiment can be completed in only 40 min. Cross-linked cells are placed on the microfluidic chip to generate sonication of DNA through a magnetic field. The use of crescent structures increases the gas-liquid contact surface, increasing the power of the magnetic field. Low acoustic waves are then used to allow greater interaction between the beads and cell extract. The immunoprecipitation step is completed after 30 min. The beads are simply washed with a buffer to remove impurities and then collected for analysis. The obvious advantages of this technique include small amounts of starting material, ease of experimental procedure, very short experiment times and high resolution.

1.2.2 Cellular Thermal Shift Assay (CETSA)

CETSA allows for the evaluation of protein interactions through macromolecular stabilization or destabilization and therefore variation in the melting temperature (Tm) [24]. Specifically, this technique is based on the biophysical principle that if a protein/enzyme is subjected to temperature increase, it will undergo protein unfolding with different melting curves. CETSA has several advantages: (1) it is not necessary to modify the molecule for immobilization on beads (as in chemo-proteomic approach, AlphaLISA, MST); (2) it is not an expensive technique; and (3) it is very simple to perform. The CETSA protocol includes the following steps: incubation of cells or cell extracts (to ensure permeability of the compound) with the molecule of interest; incubation of cell pellets at different temperatures, usually between 37°C and 70°C (the choice of temperature depends on the protein under investigation); total protein extraction; and detection of stabilization by Western blot.

Over the years, this technique has undergone various modifications, such as the use of different compound concentrations to evaluate dose-dependent enzymatic stability or to study not only the interaction of the molecule with a single protein but with all the cellular proteasomes. A two-dimensional thermal proteomic profile strategy was developed to investigate all the off-target effects of panobinostat (an epigenetic drug used for multiple myeloma) [25]. After treatment of the cells with panobinostat at five different concentrations and incubation at 12 different temperatures, all proteins undergoing a change in Tm, and therefore all proteins that bind the drug, were analysed by liquid chromatography-mass spectrometry (LC-MS)/MS. Using this assay, four new different proteins which interact with the compound were identified. One of the limitations in using CETSA is that it is unsuitable for HTS of protein interactions due to the time and quantity of materials required. In 2017, Holbert and colleagues [26] developed a new method named high-throughput dose-response (HTDR)-CETSA, which allows for HTS using a 384-well plate format of drug-protein interactions by chemiluminescence signal (Fig. 3). In this assay the target protein, SMYD3, is fused and overexpressed in the cell using the BACMam system (ThermoFisher Scientific), with a small enzyme donor fragment of β-galactosidase (β-gal) called enhanced ProLabel® (ePL; DiscoverX).

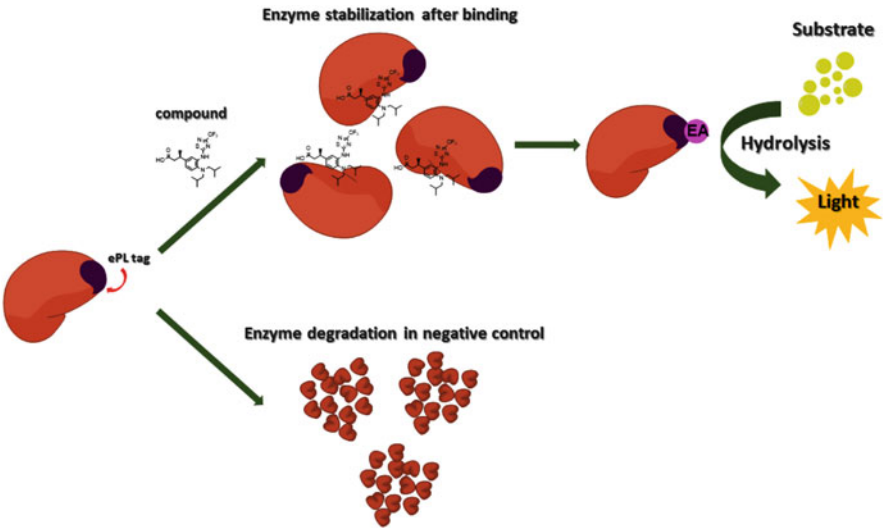

Fig. 3 CETSA in chemiluminescent system. The protein SMYD3 is expressed with ePL tag. After induction and stabilization with GSK5628, the enzyme acceptor binds ePL tag and hydrolyses the substrate

Upon addition of a compound that binds the target, stabilization can be monitored by measuring target protein abundance using chemiluminescent detection. The detection reagents are a chemiluminescent substrate added with a large enzyme acceptor fragment that reacts with the ePL tag to create an active β-gal enzyme, which hydrolyses the substrate to generate a chemiluminescent signal. The signal is directly proportional to protein stabilization (www.discoverx.com/products-applications/target-engagement-assays). The cells were plated in 384-well plates, incubated with the compound for 1 h and treated at different temperatures, as in the standard CETSA protocol. The cell reagent was added and the signal was read after incubation for 3 h at room temperature. Using HTDR-CETSA, (1) it is possible to quantify the protein interaction; (2) centrifugation and washing are not necessary because the results are not affected by the presence of serum or cellular lysate; and (3) data observation is more simple and rapid than in standard CETSA, which uses Western blotting to confirm the protein interaction.

1.2.3 Förster Resonance Energy Transfer (FRET) and Bioluminescence Resonance Energy Transfer (BRET)

FRET and BRET are two effective methods used to identify protein interactions in cells in real time by measuring the interaction between donor and acceptor chromophores. FRET is a physical assay where laser light induces donor excitation and the

transfer between excited donor and acceptor chromophores when the molecules are in proximity (100 Å) [27]. Förster proposed that the energy (k_t) was given by:

$$k_t = r^{-6}k^2Jn^{-4}k_F \times 8.71 \times 10^{23}$$

where r is the distance between donor and acceptor, k^2 is the orientation factor, J is the overlap between donor and acceptor spectrum, n is the refractive index of the medium and k_F is the ratio constant emission of donor. FRET is generally read through an imaging-based assay or flow cytometry to measure the molecular distance or the "on" and "off" interaction state. Depending on the experimental aim and the epigenetic protein under investigation, several cell types including U2OS, HEK293, HeLa, COS-7, CHO and HCT-116 cells can be used. A number of instruments are available for imaging such as Image Xpress (Molecular Devices), IN Cell Analyzer (GE Healthcare) and Operetta (PerkinElmer). Alternatively, commercial plate readers can be used to detect the FRET signal, including Infinite M1000 (Tecan), PHERAstarFS (BMG), Synergy 2 and 4 (BioTek) and Envision (PerkinElmer).

Fluorescence lifetime microscopy (FLIM) is a third alternative for measuring FRET in presence and absence of compounds. It only measures changes in donor fluorescence lifetime. Recently, FLIM microplate readers have been produced that are compatible with SBS standard labware and improved to perform 96-well plate reads of tens of minutes instead of hours (see, e.g. http://www.fluorescenceinnovations.com/cells.html) [28, 29]. Using FRET to visualize histone methylation in cells, Tinget al. developed a plasmid-based biosensor which consists of a peptide (substrate, H3 at K9 or K27) that is inserted into a flexible linker with a methyl-lysine binding domain (chromodomain), which binds selectively to lysine-methylated peptides. When methylation occurs at the histone-derived peptide, the methyl-lysine binding domain causes a structural change and induces a FRET signal. Because this mechanism is reversible, demethylation induces the separation of fluorescent units and so lowers the FRET signal back again.

FRET has two main advantages: (1) it does not use additional substrates to generate the signal and (2) it is able to measure the interaction with low protein expression better than other assays, such as BRET. However, the technique also has several disadvantages: (1) the intensity of laser light can cause problems of phototoxicity or photoheating; (2) bleed-through occurs when an acceptor is excited by the donor excitation wavelength and vice versa; and (3) there may be an overlap in donor and acceptor emissions. BRET overcomes some of the problems associated with the FRET approach. BRET is based on the same principles as FRET. The energy is generated by a fluorescent donor, usually Renilla luciferase (Rluc), which matches with yellow fluorescent protein (YFP) [30]. Rluc was originally chosen because its emission spectrum overlaps with YFP emission spectrum. It is now possible to change the Rluc and protein acceptor (Table 3).

In recent years, NanoLuc luciferase (Nluc) assay was developed to increase the distance between the donor and acceptor spectrum and to improve the low quantum

Table 3 Some protein donors and acceptors in FRET

Donor	Donor emission (k, nm)	Acceptor	Acceptor excitation/emission (k, nm)
Rluc	420–530	EYFP	513/527
RLuc	420–530	Topaz	514/527
RLuc	385–420	GFP	405/510
Aequorin	430–500	GFP	489/510

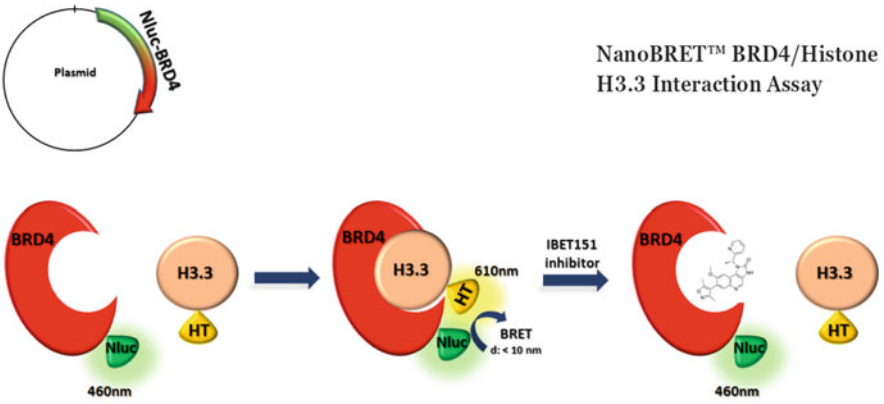

Fig. 4 NanoBRET interaction assay. BRD4 is co-expressed with Nluc with emission at 460 nm. The enzyme interacts with the substrate and the BRET signal is observed. IBET151 binds BRD4 and only the light emission of Nluc is visible

yield and poor luminescent stability of BRET [31]. Nluc uses furimazine, an engineered small protein (19 kDa), as the donor, which produces a higher luminescent signal than Rluc and allows better discrimination with acceptor fluorophores, and a fluorescently labelled HaloTag fusion protein as the acceptor. A recent study demonstrated that chloroalkane derivative of non-chloro TOM dye is the best acceptor substrate [31]. It provides peak light emission at 635 nm, and so the spectra are separated by 175 nm. Nluc is able to visualize subcellular translocation events with sub-second temporal resolution. It is in fact possible to evaluate bromodomain activity and identify compounds that inhibit it using HTS. The interaction between the bromodomain and the acetylated histone substrate can be monitored by expressing Nluc-BRD4 (one of the BET family members [32] in mammalian cells with H3.3-HT protein). When BRD4 binds H3.3 substrate, the distance between Nluc and HT is less than 10 nm and it is possible to see the signal. Treating cells with an inhibitor that competes with the substrate induces the loss of BRD4-H3.3 binding and so a decrease in BRET signal (Fig. 4). Similarly to FRET, with the BRET approach, it is possible to use a cell line that can be transfected (e.g. HEK293, HeLa, HCT-116, NIH3T3, CHO and Jurkat). It is generally better to generate a stable cell line expressing one or both proteins for a higher and clearer signal. Commercial plate readers pre-equipped with the correct filter set-up include GloMax Discover (Promega) and CLARIOstar (BMG). Instruments that can be supplied with optional

filters include Varioskan (Thermo Scientific; Edmunds Optics filters: donor 450 nm CWL, 25 mm diameter, 80 nm FWHM, Interference Filter and acceptor 1 in. diameter, RG-610 Long Pass Filter) and EnVision (PerkinElmer; Chroma filters: Emission Filter for EmSlot4).

2 Innovative Genome-Wide Technologies

Numerous accurate and reliable approaches have been gradually introduced to study epigenetic processes both at the level of single genes and genome-wide, collectively called epigenetic technologies [33]. Progress in epigenetics [34] is closely linked to the design of new technologies. Since epigenetic alterations may cause several disorders, studying the (epi)-genome is a valid approach to identify these deregulations. While the genome itself is stable, epi-modifications are in continuous transformation and so the choice of methodology is crucial. Genome-wide research technologies [35] have dramatically enhanced the study of epigenetic phenomena and the development of new epigenetic-based diagnostic and therapeutic tools. Many innovative platforms associated with advanced software packages for the study and interpretation of the epigenome are now available. These platforms are often related to specific diagnostic panels for different diseases. This section will describe the most advanced methodologies for epigenome-wide applications with a particular focus on single-cell epigenomic methodologies [36].

2.1 ChIP: From Standard Procedures to Innovative Applications

ChIP is one of the principal methodologies used to examine the epigenetic state of chromatin, that is to say the regulatory processes including direct methylation of DNA, covalent modifications to histones and protein interactions with genomic regions. The standard ChIP protocol [37] includes a set of specific steps as shown in Fig. 5. Over the years this technique has been extensively revised and improved to provide a better "resolution" of epigenetic markers and profiles. However, ChIP results can still be disappointing for several reasons: high background binding, inappropriate controls, low affinity of antibodies, errors made during DNA amplification and suboptimal quality of reagents used in chromatin preparation procedures. A further critical experimental point involves the starting material. Since ChIP comprises numerous steps, the amount of cells is crucial and pooling immunoprecipitated DNA samples is often a mandatory strategy. Sometimes pooling starting material is not always possible as specific cell types, cancer cells or heterogeneous materials in general are used. The size of DNA fragments is another key issue. DNA fragments are usually obtained by sonication and optimal fragment size

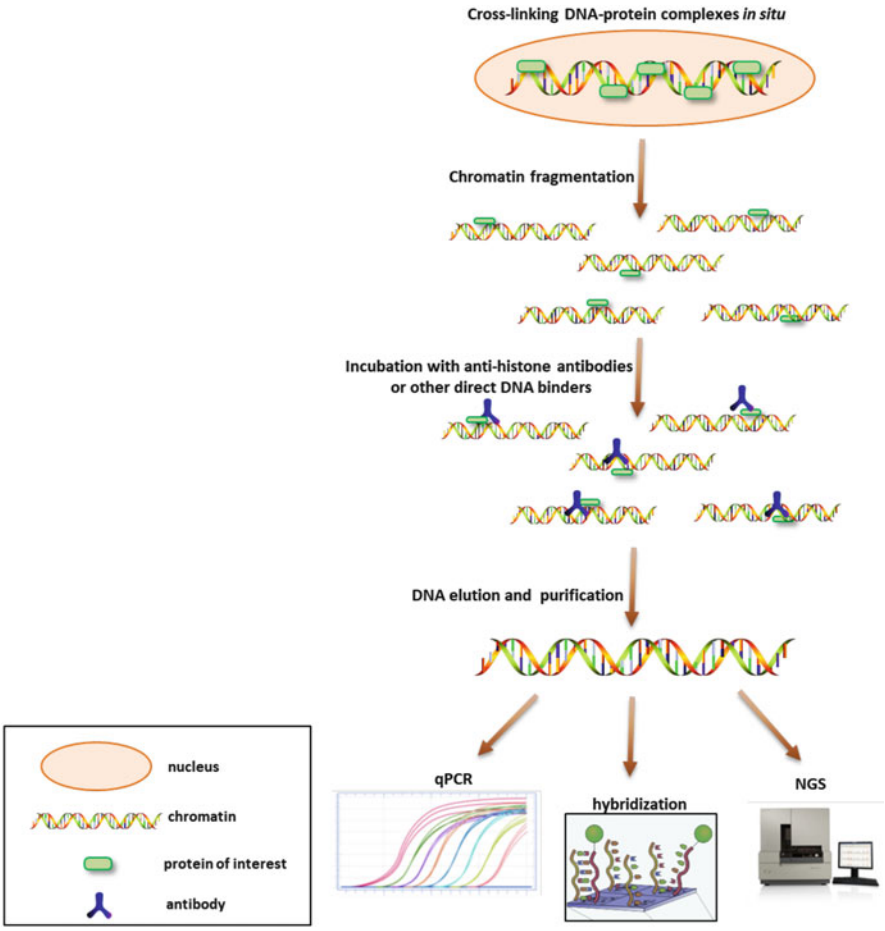

Fig. 5 Schematic representation of standard ChIP procedure. The figure shows the main steps characterizing ChIP basic protocol: DNA-protein complexes are in situ cross-linked, following chromatin fragmentation by sonication and incubation with specific antibodies. DNA is then eluted and purified. Finally the last steps include analysis by quantitative PCR, hybridization platforms or NGS (Chip-seq)

should be in the range of 200–500 bp. This is a very important step which depends on many different variables such as the kind and quantity of starting material (cell line, tissue, etc.), the power and frequency of sonication, the timing for and between each step and the sonicator itself. As previously discussed, conventional ChIP requires a huge amount of starting material, which is often lost during the numerous steps involved in the protocol. This is a crucial drawback, which often does not allow appropriate validation of the method, thus preventing the generation of reliable and precise results. As the use of personalized therapies is increasingly playing a fundamental role in the study and prevention of various diseases, new nonconventional

approaches are continuously evolving. One of the latest applications involves the use of targeted chromatin ligation (TLC), by which it is possible to start from a very small quantity of biological material [38]. This is especially important when starting material comes from biopsy specimens of human tissues or rare cell subpopulations. This alternative method preserves the sample without requiring the numerous steps involved in conventional ChIP procedures. The TLC technique increases the recovery of enriched material and improves the efficiency of ChIP using a simplified workflow. This approach excludes the use of beads by recruiting proximal binding of the antibody to specific adapters, followed by subsequent amplification of the ligated chromatin involving fewer steps and much shorter experimental times. This new application greatly reduces the methodological complexity by increasing the sensitivity of results obtained. These conditions have been optimized over time and more specific protocols have been developed.

2.1.1 ChIP in Microfluidic Droplets

This microfluidic-based method is another advanced tool able to overcome the limitations of standard ChIP described above. It involved for the first time the development of ChIP in droplets based on a microfluidic platform that combines nanolitre droplets, magnetic beads and tweezers. This innovative technique has many advantages including compartmentalization within nanolitre droplets, improved mixing, reduced consumption of samples and reagents, lower assay time (about 7 h), decreased number of cells required, lower cross contamination and very high sensitivity/specificity. The assay is very useful for the study of epigenetic processes/modifications. The protocol [39] was specifically designed to investigate four different histone modifications, with known epigenetic roles. This integrated ChIP procedure is performed using a Teflon tube platform. The system consists of a syringe pump which pushes sequential droplets across the tube toward a magnetic tweezer, whose activation/deactivation gives the extraction and dispersion of magnetic beads and droplets. The syringe pump permits droplet manipulation and processing by switching from injection to aspiration mode. The first train of droplets is made of magnetic beads associated with antibodies and chromatin, washing buffer, RNase and proteinase K. Magnetic beads are collected by magnetic tweezers and then extracted, washed in buffer droplets, transferred to the RNase droplet for RNA digestion and extracted after 5 min incubation. Subsequently, magnetic beads are dispersed in the proteinase K droplet for protein digestion and heated for 5 min at 70°C to release DNA from digested proteins. Magnetic beads are then discarded, and a new train of droplets is generated with charge switch magnetic beads, washing buffer and elution buffer droplets. These last beads are then transferred to the droplet containing DNA and digested proteins to capture the DNA, before it is extracted, washed and eluted and finally discarded before qPCR. The sensitivity of the droplet methodology is, however, highly dependent on an adequate quantity of magnetic beads as well as chromatin and specific antibodies. The choice of a well-defined range of starting material is therefore crucial to improve assay performance.

Recently, the droplet-based microfluidic system was also used by combining DNA barcoding and NGS in order to collect and compare orthogonal individual cell gene expression data [40]. This approach is crucial since epigenetic heterogeneity is often difficult to detect due to the complexity of chromatin organization and different patterns of variability. Assaf Rotem and colleagues identified the profiling of histone methylation marks (H3 lysine 4 trimethylation and demethylation) in a mixture of mouse populations of embryonic stem cells (ESCs), fibroblasts and haematopoietic progenitor cells by applying this method to discriminate and identify high-quality chromatin profiles for each cell present in the blend. Droplet-based ChIP assay thus provides a highly advantageous, rapid and cell-specific approach to epigenetic analyses.

2.1.2 G4 ChIP-Seq

G4 ChIP-seq is a novel method which can be used to determine G-quadruplex (G4) structure formation genome-wide in chromatin [41]. DNA sequences are able to form four-stranded G4 secondary structures that are involved in epigenetic regulation. This modified protocol applies standard ChIP-seq for the detection of secondary DNA structures using a specific antibody. As with any ChIP assay, the quality of the antibody used is critical. This technique uses the G4 structure-specific single-chain fragment variable antibody BG4, which is prepared using the expression vector pSANG10-3F-BG4.

Although G4 ChIP-seq is a highly innovative method that recognizes chromatin regions forming G4 DNA structures in a cell population, it presents technical limitations due to the detection of G4 structures at a given locus present in a heterogeneous cell population. In addition, this experimental approach is not able to detect the temporal organization of G4 structure and on which strand it is formed. The entire protocol requires less than 1 week to be completed and might be also combined with a microfluidic method (described in the subsection above) for single cells in order to avoid some of the issues linked to heterogeneous cell populations. Despite technical limits, the use of this method led to the identification of restricted regulatory regions of chromatin, focusing specifically on regions regulating cell fate and function [41] which are not often detectable by standard procedures. The next step will be to apply this technology in human tissues and other model organisms.

2.1.3 Standard Epigenome-Wide Association Studies (EWAS)

New high-throughput genome-wide epigenetic technologies such as ChIP-seq [42] and ChIP-on-chip [43] have recently been developed. ChIP-on-chip arrays are available from Affimetrix, Agilent and NimbleGen, in both whole-genome tiling array format for model organisms including human and mouse, as well as focused arrays for promoter regions and custom array designs. The main advantage of ChIP-on-chip is the huge amount of data generated which can be statically analysed

minimizing the artefacts and improving the significance and resolution of results. EWAS are designed to identify a specific epigenetic mark [44] associated with a particular phenotype through the use of array and sequencing-based technologies.

One of the most studied epigenetic marks is DNA methylation of 5-methylcytosine (5mC) on CpGislands [45] since methylated DNA is the key component of epigenetic information in mammals [46]. A first step in studying DNA methylation is to quantify all global levels of DNA methylation. Massive parallel sequencing (MPS) [47] quantifies results after DNA pretreatment. Pretreatment may be affinity-based, which enriches methylated or unmethylated sites of DNA. Alternatively, pretreatment may be performed using methylation-sensitive restriction enzymes or bisulfite conversion of DNA. In the latter case, the gold standard approach is whole-genome bisulfite sequencing [48], which provides quantitative information about cytosines throughout the genome. However, this is a very expensive assay.

MPS approaches can be divided into two different groups: (1) reduced representation bisulfite sequencing (RRBS), which uses a measuring range of generated fragments from digestion with restriction enzymes for targeting deep sequencing at specific loci, and (2) HELP-tagging assays using digestion with methylation-sensitive restriction enzymes in order to generate tags at these sites, which represent the DNA methylation level. MPS provides data that cannot be obtained by microarray such as single-nucleotide polymorphisms. As microarrays are designed to recognize 5mC, in the context of CG, in some cases, levels of CHG and CHH (where H equals A, T or C) may not be detected or might interfere with restriction enzyme digestion. In bisulfite sequencing (BS-seq), cytosine that is not converted by bisulfite can be both 5mC and 5-hydroxymethylcytosine (5hmC). In order to overcome this issue, two different arrays are therefore necessary. Four different modifications including 5hmC, 5mC, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) are read by bisulfite as unmethylated cytosine, which could require more specific assays [49], described in the following subsections. Although these assays have been improved to be specific for each type of modification, considering the huge amount of (epi)-genetic modifications underpinning different areas of the genome, they inevitably present both advantages and disadvantages (Table 4).

2.1.4 Tet-Assisted Bisulfite Sequencing (TAB-Seq)

TAB-Seq is a novel method using bisulfite conversion and Tet proteins to study 5hmC [50]. Specifically, genomic DNA is first treated consecutively with T4 phage β-glucosyltransferase (β-GT) and recombinant mouse Tet1 to convert 5hmC to 5gmC and 5mC to 5caC. β-GT catalyses the attachment of β-D-glucosyl residues from uridine diphosphoglucose to the hydroxyl group of 5hmC. DNA bisulfite treatment leaves 5hmC unchanged, while 5mC and unmethylated cytosines are converted to uracil. This assay provides an accurate representation of 5hmC localization in the genome and clearly differentiates between 5hmC and 5mC, specifically

Table 4 Advantages and disadvantages of different ChIP-seq applications

Method	Advantages	Disadvantages
TAB-seq	Accurate representation of 5hmC localization	Detection limit for the quantification of 5hmC
EnIGMA	To decipher the sequence converted by bisulphite back to the original sequence	DNMT1 enzyme does not methylate hemi-hydroxymethylated CpGs and non-methylated CpGs
CAB-seq	Detection of 5caC with single-base resolution in DNA	Low protection rate of 5caC deamination
fCAB-seq	Identification of 5fC using BS-seq	Useful for base-resolution mapping
scBS-seq	To avoid the loss of genomic DNA in individual cells during DNA purification	DNA degradation after bisulfite step
scRRBS	To provide genome-wide coverage of CpGs in islands at single-base resolution and of dense areas in CpG methylation	Biased sequence selection
snmC-seq	Gain insights into epigenetic alterations and regulation	Broader applications

identifying 5hmC. Further, CpG and non-CpG hydroxymethylation throughout the genome is covered at single-base resolution. This is a very accurate method, but large amounts of DNA starting material are required and the detection limit is insufficient for the quantification of 5hmC with extremely low 5hmC levels. To date, many other methods have been developed and improved such as those for the direct identification of mC, hmC and unmodified cytosine (C) at single-base resolution.

2.1.5 Enzyme-Assisted Identification of Genome Modification Assay (EnIGMA)

EnIGMA is a highly efficient and reliable analytical method based on the specificity of the enzyme DNMT1 [51]. Specifically, the enzyme methylates the cytosine of hemi-methylated CpGs but does not methylate hemi-hydroxymethylated CpGs and non-methylated CpGs. The technique includes genomic DNA digestion with the appropriate restriction enzyme, followed by digested DNA end-repairing and A-tailing. Ligation of hairpin-shaped adaptor DNA with deoxyuridine is then followed by "USER" enzyme digestion. Alternatively, digested DNA is dephosphorylated and directly ligated with hairpin-shaped adaptor DNA using the cohesive end of the restriction enzyme cutting site. DNA is then treated with DNA polymerase and DNMT1 enzyme. Finally, the method includes bisulfite treatment and PCR. Sequencing and CpG comparison are necessary to determine whether the cytosines in the original DNA were mC, hmC or unmodified C. The procedure can be applied to many types of epigenetic studies including comprehensive genome-wide analysis of hmC using massive parallel sequencers. This method is also very useful to decipher the original sequence converted by bisulfite, eliminating the need

for re-sequencing. The technique has recently been used in studying genes involved in mouse brain development such as Arhgap27 and Nhlrc1, which have a significant number of hmC associated with single-nucleotide polymorphisms. EnIGMA is also important in the study of ESC memory, since cytosine modifications control epigenetic status across the epi-(genome) [51].

2.1.6 Chemical Modification-Assisted Bisulfite Sequencing (CAB-Seq)

CAB-Seq can detect 5caC with single-base resolution in DNA. In this technique, 5caC is selectively labelled by 1-ethyl-3(3-dimethylaminoproyl)-carbodiimide hydrochloride (EDC) by using a xylene-based primary amine [52]. This specifically labels 5caC but no other modified cytosine. This modified 5caC does not undergo deamination by bisulfite as does unmodified 5caC and is therefore sequenced as a cytosine instead of a thymine. However, this technique has a low protection rate of 5caC deamination (about 50–60%) and requires the sequencing of a non-treated DNA control. The use of this technique involves mouse ESCs, which are characterized by a strongly decreased quantity of 5caC.

2.1.7 5-Formylcytosine Chemical Modification-Assisted Bisulfite Sequencing (fCAB-Seq)

fCAB-seq [53] is a chemical method to identify 5fC using BS-seq. The technique exploits the reaction of hydroxylamine with 5fC but is limited by the fact that it is only useful for base-resolution mapping. In fCAB-seq, 5fC is protected by bisulfite-mediated deamination by treatment with O-ethylhydroxylamine. Hydroxylamine-protected 5fC is therefore read as a cytosine instead of a thymine, as in BS-seq. By comparing data from BS-seq and fCAB-seq, the locations of 5fC from unmodified cytosine and 5CaC can be differentiated. RRBS for 5fC is based on the chemical selective reduction of 5fC and subsequent bisulfite conversion. After bisulfite treatment, 5fC is deformylated and subsequently deaminated in uracil, so that it is read as a thymine and identified as an unmodified cytosine in BS-seq. This method has also been used in a mouse ESC model in which genomic distribution and the DNA glycosylase TDG strongly depend on 5fC regulation. 5fC profiling is further associated with 5mC/5hmC oxidation at different gene regulatory elements. In addition, 5fC accumulation correlates with p300 binding at poised enhancers, thus regulating important epigenetic mechanisms [53].

2.1.8 Single-Cell Bisulfite Sequencing (scBS-Seq)

This genome-wide DNA methylation sequencing method can also be reproduced in single cells by integrating some of the steps in the standard RRBS protocol in order

to avoid the loss of genomic DNA in individual cells during DNA purification [54]. This method is useful for the epigenetic study of heterogeneous and/or rare cell populations. The approach has been applied in mouse ESCs used as a model of study due to their versatility, allowing identification of epigenetic and cellular biological heterogeneity [55]. The disadvantage of this procedure is that DNA may undergo degradation after the bisulfite step. This method has been improved in combination with single-cell genome and transcriptome sequencing in order to explore novel correlations between epigenetic modifications and gene expression in embryonic stem cells. The protocol has been modified with particular regard to amplification steps. Further, this method was used to study methylated DNA heterogeneity in a cell population in order to understand the role of this epi-mark in a well-defined developmental process of disease compared to normal conditions [56].

2.1.9 Single-Cell Reduced Representation Bisulfite Sequencing (scRRBS)

In the scRRBS method, genomic DNA is previously digested with one or multiple restriction enzymes (e.g. MspI) that identify common sites on CpG islands (CCGG sites) to produce sequence-specific fragmentation [54]. The fragmented genomic DNA is then treated with bisulfite and sequenced. This approach has the advantage of providing genome-wide coverage of CpGs in islands at single-base resolution as well as coverage of dense areas in CpG methylation. The disadvantage of conventional RRBS is biased sequence selection due to the fact that restriction enzymes cut at specific sites. In addition, the assay measures 10–15% of all CpGs in the genome and is not able to discriminate between 5mC and 5hmC. Differences from the standard method include the biotinylated 5′ end of universal adapters and 3′ end of index adapters and the introduction of rescue steps. In this way, it is possible to increase DNA sequences by PCR.

2.1.10 Single-Nucleus Methylcytosine Sequencing (snmC-Seq)

snmC-seq is a high-throughput method based on a whole-genome bisulfite sequencing approach to identify differentially methylated regions across thousands of cells in order to elucidate cellular diversity of complex tissues [57]. This high-throughput single-cell sequencing technique can be useful to gain insights into epigenetic alterations and regulation. The method has been applied in human and mouse frontal cortex neurons to identify specific neuronal clusters through the use of specific neuronal markers followed by correlation analyses [57]. Each cluster revealed differentially methylated regions characterized by low mCG in defined neuronal populations. Technical application on single neuron epigenomic profile therefore identified distinct mC regions containing crucial information for specific marker regions and regulatory elements both in human and mouse.

2.1.11 Single-Cell Genome-Wide Methylome and Transcriptome Sequencing (scM&T-Seq)

scM&T-seq is a recently developed method for the study of parallel single-cell genome-wide methylome and transcriptome sequencing, which provides information on transcriptional and epigenetic heterogeneity [58]. scM&T-seq uses Smart-seq2 and scBS-Seq to determine DNA methylation patterns. This approach is useful because there is no need to mask coding sequences during analysis since DNA and RNA are independently amplified. However, the technique has two main drawbacks: (1) it is impossible to distinguish between 5mC and 5hmC and (2) Smart-seq2 is not strand-specific and is applicable to only poly(A)-RNA. This technique provides a very important approach to the analysis of cellular plasticity and heterogeneity. Applications involve the use of mouse ESCs characterized by a strong heterogeneity state and variability. Using scM&T-seq it was possible to study these conditions at single-cell level by NANOG-low and REX1-low cells in sorted populations. NANOG and REX1 are in fact transcriptional factors involved in ESC differentiation, pluripotency and renewal [58], and this type of approach is of crucial importance to detect methylated distal regulatory elements associated with transcription of these key pluripotency genes.

2.1.12 Three-Dimensional (3D) Chromatin Methods

The architecture of chromatin is characterized by various levels of complexity that define the fate of specific genes in terms of regulation and expression, as well as regulatory regions. Thus, understanding the association of chromatin with specific proteins such as histones through 3D interactions is highly desirable. Many cutting-edge techniques have been developed to decipher specific genome areas as a "photo shot". These technologies involve chromosome conformation capture (3C) and 3C-based methods which decode the 3D organization of the genome and its structural conformation at specific loci. The 3C and 3C-derivative methods [59] offer the advantage of studying the genome from specific areas up to its entirety both in terms of complexity and function. The steps of these methods include those already used in standard ChIP assays, but unlike ChIP, they are able to identify ligation junctions between genomic loci. The protocol involves chemical cross-linking with formaldehyde and subsequent chromatin purification. Subsequently, specific restriction enzymes are used that are able to recognize interactions in DNA. Once chromatin fragments are released, they are further ligated and cross-linking is reversed. The final step is real-time PCR analysis, which recognizes the ligase product from the previously isolated DNA, providing a quantitative response of the interaction frequency between two ligated DNA regions. One of the techniques deriving from 3C is 4C, which allows the detection of an unknown region of DNA that interacts with a specific locus of interest. Unlike the 3C method, 4C uses a second restriction

enzyme, which recognizes a different sequence from the first in order to recirculate the DNA of interest linked to its interacting DNA. 4C technology is an advanced 3C-based approach, as it combines 3C with NGS technology. A further variant of 3C is 5C technology, which has the added benefit of studying millions of interactions in genomic areas by using universal primers in one single assay. 3C-based technologies include Chromatin Interaction Analysis by Paired-End Tag Sequencing (ChIA-PET), which combines the 3C method with ChIP and examines genomic sites associated with specific transcription factors, which can be proximal and distal [60]. In ChIA-PET, a specific antibody is used together with sonicated chromatin and the protein of interest, and only this immunoprecipitated material linked to the protein of interest is used to construct the ChIA-PET. Then, biotinylated oligonucleotides are used to ligate DNA proximal fragments, which are subsequently ligated on beads. Finally, DNA is digested by a specific restriction enzyme which cuts to release a tag-linker-tag region. After pull-down of biotinylated linkers by specific beads, DNA tags are amplified. Hi-ChIP is an advanced Chia-PET method [61] which provides a more accurate interpretation of information on genome conformation. In Hi-ChIP, DNA interactions are firstly established in situ in the nucleus before lysis procedure. In this way it is possible to avoid false-positive interactions.

3 Epigenetic Regulatory Role of miRNA: New Analytical Tools and Technologies

miRNAs are small non-coding RNAs whose role as actors and targets in epigenetic machinery is well established. They are efficient regulators of heterogeneous physiological and pathological functions, as is well described in almost every cancer model. The clinical potential of miRNA lies in their fundamental tumour-suppressive and oncogenic functions and in their ability to modulate the efficacy of cancer treatments, such as chemotherapy and radiotherapy. miRNAs have proven to be efficient clinical diagnostic and prognostic markers in several human diseases including cancer, as well as therapeutic targets of biological and chemical molecules. Studies on selective miRNA modulation through antisense inhibition, mature miRNA replacement, chemical modification for better delivery or chemical compounds could have a significant impact on the successful translation of miRNA-based or miRNA-targeted therapeutics from bench to bedside. In recent years, different experimental and computational approaches using methodologies associated with miRNA detection and bioanalysis have therefore experienced rapid growth. This section provides an overview of miRNA research methods and technologies, mainly focusing on the latest and most advanced miRNA analysis strategies.

3.1 *miRNA Quantification Techniques*

qPCR, microarray and NGS are the most commonly used methods for miRNA quantification and profiling. They provide sensitivity and reproducibility and have become more cost-effective and easier to perform as a result of improved strategies and an increase in their global application.

3.1.1 qPCR

qPCR is currently the gold standard for miRNA quantification. Basically, miRNAs are amplified with reverse transcription (RT)-PCR to generate cDNA, and the miRNA of interest is then amplified and quantitatively measured in real time using fluorescent probes. SYBR Green and TaqMan (ThermoFisher Scientific) are two widely used systems. Although TaqMan technology offers greater miRNA detection sensitivity and specificity, qPCR procedures should avoid primer dimers and increase the sensitivity of detection threshold. Stem-loop RT [62, 63] and locked nucleic acid (LNA) primers [64] were developed to overcome these limits. Stem-loop RT-PCR is a new real-time RT-PCR for miRNA quantification. It includes two steps: RT and real-time PCR. First, stem-loop RT primers bind to the 3′ portion of miRNAs and are reverse transcribed by reverse transcriptase. cDNA is then quantified using conventional TaqMan PCR, which includes miRNA-specific forward primer, reverse primer and dye-labelled TaqMan probes (Fig. 6a). In this way, miRNA quantification shows

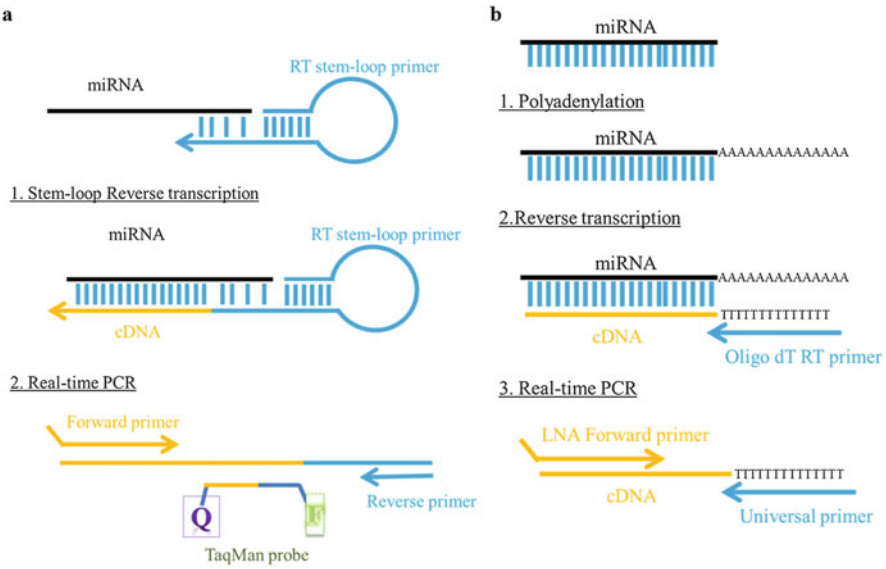

Fig. 6 Schematic representation of real-time RT-PCR for miRNA quantification by stem-loop RT (**a**) and LNA primers (**b**)

very high specificity, without false positives, and is sensitive for low amounts of these short RNA targets (such as 10 pg of total RNA). In the LNA assay, the RT-PCR reaction is performed with LNA primers. LNA is a modified RNA nucleotide in which the ribose ring is "locked" in the ideal conformation for Watson-Crick binding. LNA-enhanced oligonucleotides improve sensitivity and specificity of RT-PC, increasing the affinity for its DNA or RNA complementary oligos, mainly for small or highly similar targets. Thermal stability considerably enhances the Tm of the duplex, which increases by 2–8°C. In addition, LNA primers are efficient at discriminating single-nucleotide mismatches. In LNA RT-PCR, miRNAs are reverse transcribed from total RNA using miRNA-specific RT primers; reverse-transcribed miRNAs are then amplified using an LNA-enhanced PCR primer together with a universal PCR primer (Fig. 6b).

LNA oligos are also used in hybridization-based technologies including qRT-PCR platforms (e.g. Qiagen), microarray and in situ hybridization (ISH). For quantification of circulating miRNAs, new technologies such as droplet digital PCR (ddPCR), Ligo-miR and Two-tailed RT-qPCR have recently been introduced to overcome miRNA normalization in biofluids and to detect even single copies with high repeatability. The ddPCR method determines absolute miRNA expression and is based on partitioning of the sample into thousands of discrete, volumetrically defined, water-oil emulsion droplets. PCR amplification of the template molecules occurs in each individual droplet using reagents and workflows similar to those used for most standard TaqMan probe-based assays. Following PCR, each droplet is analysed by estimation of the number of molecules in the reaction under the assumption of a Poisson distribution. Results are expressed as target copies per microlitre of reaction [65, 66]. The high cost and complexity of ddPCR make it difficult to screen large numbers of samples across large panels of miRNA. Ligo-miR is a multiple assay technology to rapidly measure absolute miRNA copy numbers from a wide range of biological sources. Ligo-miR assay comprises two sequential ligation steps: capture and coding (Fig. 7). Firstly, a DNA universal adapter is ligated to the 3′ end of all miRNA molecules. Then in the coding ligation up to 26 miRNA-specific discrimination probes and an Alexa647-labelled common probe are hybridized to the miRNA templates to form a single-stranded DNA product. Thermal cycling is used to perform a 50X linear amplification by repeatedly generating Ligo-miR products from each miRNA template. The products can be identified and quantified using common DNA sizing methods such as electrophoresis (denaturing PAGE). Each band is a specific miRNA product where band intensity is proportional to quantity. Absolute copy numbers can be determined using the included spike-in controls and running reference samples. The sensitivity of Ligo-miR is as low as 20 copies per cell at 75 ng input levels [67]. Two-tailed RT-qPCR is a very recent system for the quantification of miRNAs (Fig. 8). In the RT step, two specific primers about 50 nucleotides long and complementary to the miRNA target are used, while the cDNA is amplified by qPCR using two target-specific primers and SYBR Green chemistry [68].

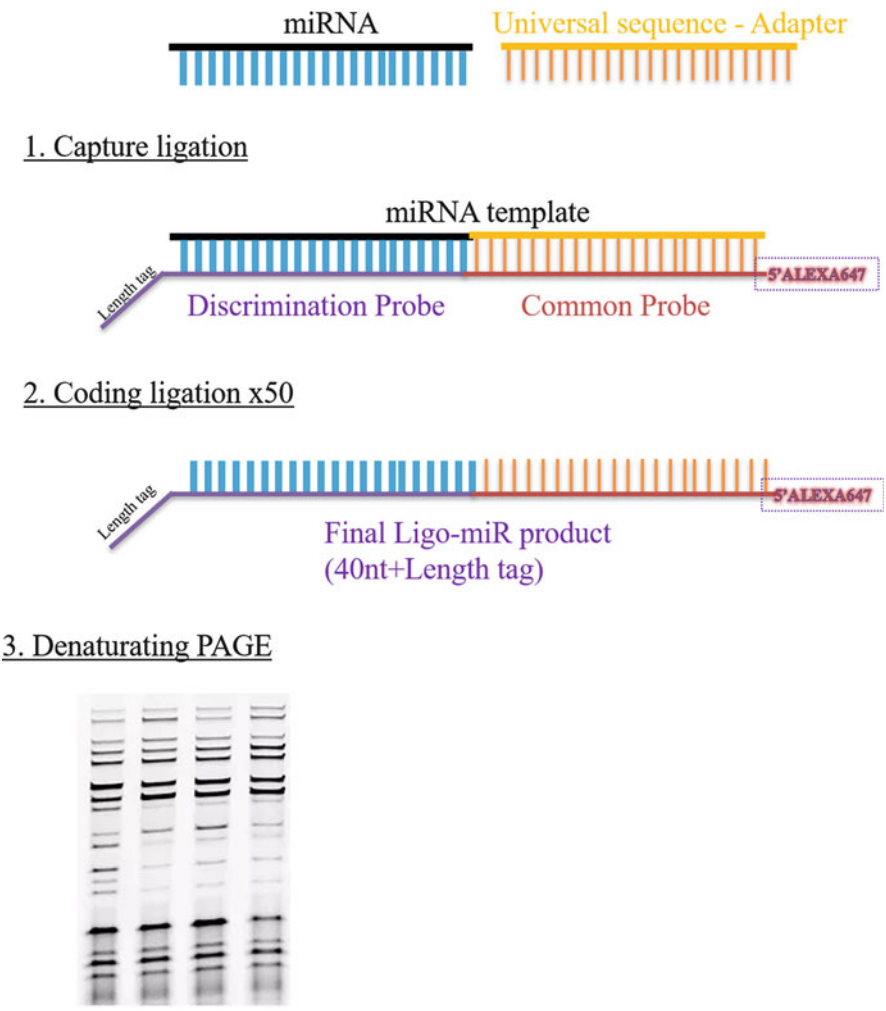

Fig. 7 Schematic representation of miRNA quantification by Ligo-miR

3.1.2 Microarray

Genome-wide analysis of miRNA expression can be performed by microarray. Microarray technology is a robust high-throughput method used to analyse simultaneously the expression of thousands of small non-coding RNAs, including miRNAs. Currently, a wide range of commercial platforms based on different technologies are available for global miRNA expression profiling, such as oligonucleotide microarray, LNA arrays (Exiqon), bead-based technology (Illumina) and microfluidic systems (Agilent, LC Biosciences). All these platforms have designed probes specific for mature miRNA sequences. The main differences are in the hybridization phase,

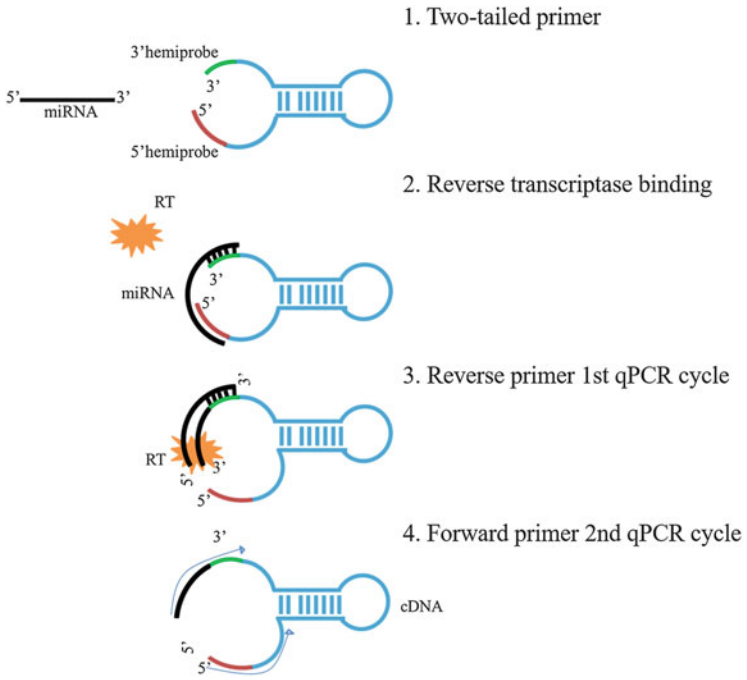

Fig. 8 Schematic representation of miRNA quantification by Two-tailed RT-qPCR

washing procedures and fluorescent dyes. The usefulness of these techniques in the clinic is limited owing to their high cost and poor reproducibility [69].

3.1.3 Next-Generation Sequencing

NGS, or second-generation sequencing, is characterized by high throughput and low cost. Currently, the most widely used sequencing systems for NGS are 454 (Roche), Ion Torrent sequencing platform (Life Technologies, Invitrogen), Illumina Genome Analyzer (Illumina) and SOLiD (Life Technologies Corporation). The basics of miRNA NGS are the same as in DNA or RNA sequencing. The NGS procedure for miRNA sequencing is divided into four phases: library preparation, sequencing, data analysis and biological interpretation. However, the various systems differ in some aspects of library preparation, such as enrichment by gel electrophoresis and choice of adapters/primers, and in data analysis. Sets of tools are available for the analysis of miRNA expression profiles, including CAP-miRSeq [70], mirTools 2.0 [71], sRNAtoolbox [72] and miARma-Seq [73]. In miRNA profiling, NGS technologies present additional advantages compared to microarray technologies, such as:

- High resolution: identification of single-base variants in the miRNA sequence.
- High throughout: de novo miRNA can be discovered.

- High accuracy: high-depth sequencing guarantees that every base is sequenced many times.

The development of high-throughput NGS methods to study miRNA expression profiles has exponentially increased the amount of data generated in the field of miRNA, bringing interesting applications in diagnostic and therapeutic approaches. Characterization of the entire miRNA spectrum by NGS provides an effective method to analyse limited sources, such as tumour biopsies from sites of difficult access, poor quality samples or circulating/exosomal miRNA from biological fluids [74–77].

3.1.4 Isothermal Exponential Amplification Reaction (EXPAR) and Advancements

EXPAR is an interesting miRNA analysis method based on isothermal amplification with high efficiency (10^6–10^9-fold). Galas and co-workers developed the EXPAR method using a combination of polymerase strand extension and single-strand nicking [78].

The basic reaction involves two steps: miRNAs hybridize with target primes and extend along the template in the presence of polymerase, and the nicking enzyme distinguishes the recognition site of double strands to cut off and generate a short DNA trigger. In the amplification phase, DNA polymerase extends the sequence to include a nicking enzyme recognition site. A nicking enzyme can then cut one of the double strands of DNA previously synthetized by DNA polymerase, which produces additional trigger sequences. This step is repeated several times usually using SYBR Green as the label. To date, different strategies have been proposed to improve EXPAR performance. Recently, an advanced miRNA assay based on the two-stage EXPAR and a single-quantum-dot (QD)-based nanosensor was developed. Importantly, EXPAR products were specifically hybridized with capture and reporter probes to form sandwich hybrids. These sandwich hybrids are collected on the surface of 605 nm emission QDs (605QDs), binding to reporter oligonucleotide/Cy5. Expression of the miRNA of interest can be analysed by Cy5 signals at excitation wavelength of 488 nm [79]. Another study proposed hairpin probes which are unfolded through catalysed hairpin assembly with the universal triggers as the primers [80]. Following a different approach, EXPAR was combined with high-resolution capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) analysis. This modified EXPAR method involves the generation of signal barcodes labelled with a fluorescent dye to simultaneously analyse the amplified products via CE-SSCP. GeneMapper v4.1 (Applied Biosystems) is the leading software used to analyse the peak of interest obtained by electrophoretic mobility [81].

Table 5 Steps and materials required for miRNA detection by ISH

Phase	Standard	Alternative options
Fixation and permeabilization	Ethanol; methanol; paraformaldehyde	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC); no proteinase K
Hybridization	DNA; RNA-probes; formaldehyde buffer	Urea-based buffer. Probes: LNA/DNA; 2′OmeRNA; LNA/2′OmeRNA; morpholino; 2′F-RNA/DNA; padlock; circular; seal; Ultramar
Washing	Formamide; saline-sodium citrate (SSC)	Tetramethylammonium chloride (TMAC) + RNase A
Sequence amplification		Rolling circle amplification (RCA); oligo-fluorescence in situ hybridization (O-FISH); RT in situ PCR

3.1.5 In Situ Hybridization (ISH)

ISH is the only method able to provide insight into both miRNA level and localization in single cells. ISH can be divided into chromogenic enzyme-based in situ hybridization and fluorescent in situ hybridization (FISH). miRNA ISH is technically difficult due to miRNA features such as small size, sequence similarity and low expression levels. The standard ISH protocol has been variously modified to improve miRNA detection in several cell lines and tissues (Table 5). The first step is cell fixation and permeabilization. The former preserves the number and localization of small RNA molecules, while the latter improves cell and tissue diffusion of the probes. Permeabilization should not be strong as it can cause RNA loss. The second step is hybridization, with optimal hybridization temperatures depending on the probe. Probe design is a key step in ISH. There are linear probes directly labelled with fluorophore or ligand and probes that enable sequence amplification. Several modifications have been proposed to enhance their binding affinity to target sequences. The third step in the ISH protocol is the washing step, used to preserve the probe-target complex and eliminate off-target binding. Finally, signal detection is a critical step to detect miRNA subcellular distribution. Non-radioactive haptens, combined with probes, are commonly used and are detected by histochemical enzymatic reactions with enzyme-conjugated anti-hapten antibodies. Different controls (such as scrambled probes, and positive and negative controls) need to be used to guarantee specificity, adequate experimental conditions and good RNA quality [82].

3.2 Experimental miRNA Target Identification

To date, the majority of miRNA target identification approaches have been based on several experimental technologies, normally combined with overexpression/inhibition of the miRNA and followed by downstream gene expression or proteomic analysis [83]. Generally, miRNA overexpression can involve the transient transfection of a

specific miRNA mimic or the stable introduction of a miRNA expression construct by a lentiviral vector. Conversely, miRNA inhibition can be achieved by chemically modified RNA analogues, such as anti-miRs [84], antagomiRs [85] and miRNA sponges [86], able to bind and block mature miRNAs. Reporter assay is used to validate individual miRNA:mRNA interactions, measuring the activity or expression of reporter protein [87]. Various computational and pull-down methods have been developed to improve the identification of the direct association of miRNA-target hybrids, including RNA immunoprecipitation of ectopically expressed components of the RNA-induced silencing complex or the affinity purification of synthetic miRNAs of interest, although the physiological significance of most identified miRNA-target associations is not still well understood [88]. Interestingly, newly developed techniques such as CLIP [89] and photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) [90, 91] investigate the indirect relationships between miRNAs and their targets, combining the stabilization of protein-RNA complexes by ultraviolet (UV)-induced cross-linking with HTS (Fig. 9). Specifically, the CLIP method is able to define interaction sites by isolating and sequencing of small RNA segments cross-linked to RNA binding proteins (RBPs) [92]. However, this approach has some limitations, such as low RNA-protein cross-linking efficiency by exposure to 254 nm UV light and high background noise, requiring several control cross-linking experiments to correctly detect the isolated RNA fragment. Some of these problems were addressed by PAR-CLIP [90]. The protocol includes the incubation of cells with medium enriched with a photoactivatable nucleoside, such as 4-thiouridine and 6-thioguanosine, improving UV cross-linking [93, 94]. Irradiation of the cells by UV light of 365 nm, which is less harmful than 254 nm for in vivo experiments, triggers the cross-linking of photoreactive nucleoside-labelled cellular RNAs to interacting RBPs. Thus, PAR-CLIP is able to enhance RNA recovery and specificity of cross-linking, achieving single-nucleotide resolution of the binding site [95]. These approaches provide a large amount of molecular information on miRNA targets identified in RBP complexes, but the detailed protocol is time-consuming and costly and requires very specialized data analysis tools. In addition, the identification of miRNAs regulating specific mRNAs remains limited due to the imperfect complementarity of miRNA:mRNA transcripts. Interestingly, miRNA Trapping by RNA in vitro affinity purification (miR-TRAP) attempts to overcome this limitation, providing an evolved in vitro RNA affinity purification protocol for the rapid capture of the complex containing the trapped miRNA/target mRNA pair in the specific cellular context of choice [96]. The advanced miR-TRAP protocol includes photoactivatable ribonucleosides on transfected miRNA to allow only for complexes containing specific miRNAs and higher wavelength cross-linking, which is preferable for in vivo studies [96]. Specifically, the miRNA is conjugated to psoralen to produce a highly photoreactive probe. When the cells are exposed to UVA light, the Pso moiety of the miRNA reacts with uridine on target mRNAs. Pso-modified miRNA mimics act similarly to endogenous miRNAs, eliminating the use of antibodies, which can create non-specific background signals and complicate data interpretation. The cross-linking between the tagged miRNAs and target mRNAs is then stringently purified by streptavidin-coated beads,

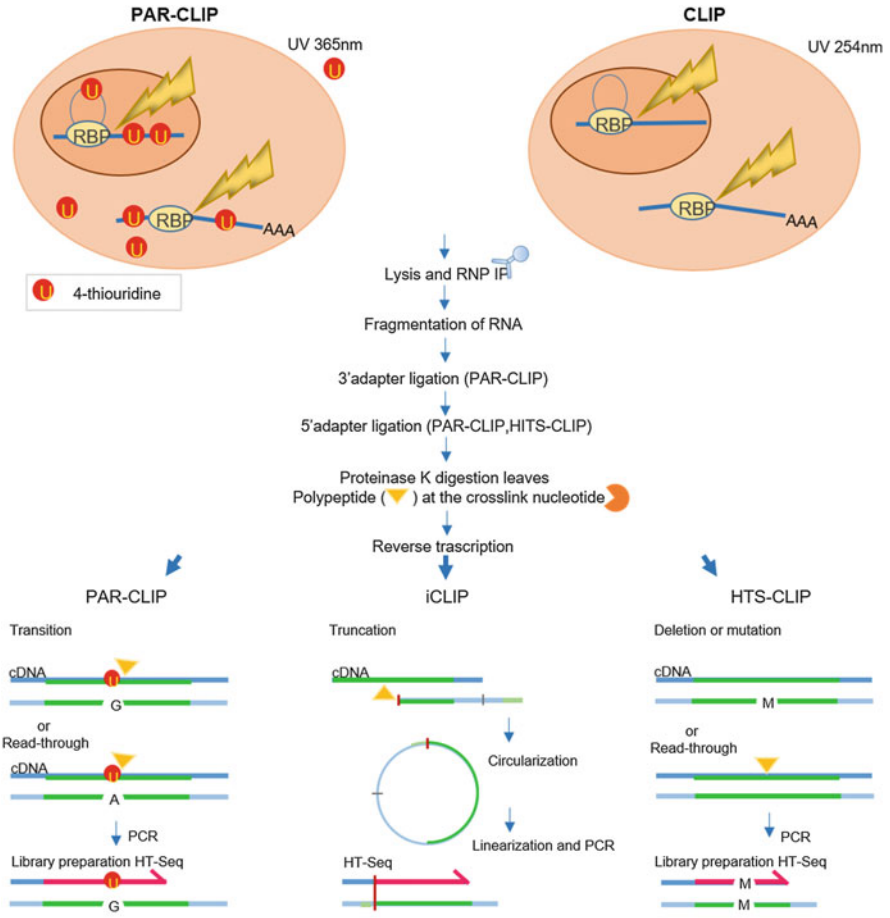

Fig. 9 Schematic representation of CLIP and PAR-CLIP procedures

minimizing the recovery of non-specific targets [96]. The pull-down method significantly enhances the enrichment of a specific target sequence for analysis via qRT-PCR. Most notably, miR-TRAP could be combined with HTS, providing the rapid identification of canonical but more importantly also of non-canonical and thus unpredictable regulatory miRNAs:mRNA networks in a cellular context [97]. This enhanced capacity has exceptional potential to discover novel mRNA targets for a miRNA of interest, including those that are transient or in low abundance. Accordingly, miR-TRAP is advantageous for the rapid identification of miRNAs at low false-positive and presumably low false-negative rates. Moreover, this approach is feasible and independent of genetic manipulations, allowing the analysis of several primary samples. Finally, miR-TRAP is a powerful, rapid, cost-effective and easy-to-handle tool that promotes the study of miRNA-dependent regulation in different diseases.

4 Conclusions

The potential reversibility of epigenetic modifications is a stimulating prospect for the development and improvement of technologies and methods to enable their characterization and, above all, to test the ability of new drugs to revert their pathological alterations, primarily in cancer. This chapter provides an overview of the current status of epigenetic assays based on epigenetic enzymatic activity and binding, genome-wide modifications and miRNAs and discusses the advantages, disadvantages and practical applications of each technique.

The growing amount of achievable information provided by innovative epigenetic approaches has prompted the parallel growth of so-called "computational epigenetics". A considerable number of computational approaches and tools are used in epigenetic studies for (1) epigenetic data repositories (e.g. International Human Epigenome Consortium Data Portal, Epigenome Atlas, Chromatin: 4DGenome, NIH Roadmap Epigenomics, miRbase, UCSC MethBase) and (2) statistical data analysis, prediction and visualization (e.g. Bioconductor (R) packages, epiGbs, MethPipe, MOABS, DaVIE, miRSystem) and data annotation, visualization and integration (e.g. DAVID, STRING, oPOSSUM, GeneMANIA, IPA).

Despite the plethora of accurate experimental and computational approaches, more efficient assays, bioinformatics methods and tools are still required to evaluate the complexity of epigenetic mechanisms and the sophisticated interaction networks governing gene expression in physiological and pathological conditions. Translating epigenetics into the clinic is undoubtedly a fundamental research perspective and the future direction of personalized medicine.

Compliance with Ethical Standards

Funding: The authors acknowledge AIRC17217; PON_0101227; VALERE: Vanvitelli per la Ricerca; Regione Campania lotta alle patologie oncologiche: iCURE (CUP B21C17000030007); and Regione Campania FASE2: IDEAL (CUP B53D18000080007). We thank C. Fisher for linguistic editing.

Conflict of Interest: The authors declare no competing interests.

Ethical Statement: This article does not contain any studies with human participants or animals performed by any of the authors.

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