METHOD FOR TREATING TISSUE SAMPLE AND METHOD FOR HIGH-THROUGHPUT TRANSCRIPTOME SEQUENCING OF SINGLE-CELL SUBCELLULAR STRUCTURE

TECHNICAL FIELD

[0001] The present invention belongs to the technical field of biology, and particularly relates to a method for treating a tissue sample and a method for high-throughput transcriptome sequencing of a single-cell subcellular structure.

BACKGROUND ART:

[0002] High-throughput single-cell nucleus transcriptome sequencing has been a relatively popular research field in recent years and has a very broad range of application scenarios.

[0003] In the snRandom-seq method of Chinese patent applications CN 202210174619.9 and CN 202210550351.4, the nuclei of the tissue are isolated, and the nuclei are fixed with formaldehyde to cross-link nucleic acids (DNA and RNA) and proteins. Then, the nuclei are used as vessels to perform a reverse transcription reaction, wherein reverse transcription primers can bind to RNA for reverse transcription to produce cDNA, and the newly synthesized cDNA and the RNA complementary strand are cross-linked together within the nuclei. Then, a capture adapter is added at the end of the cDNA. The nuclei are collected and subjected to single-cell nucleus partitioning using a microfluidic system, wherein a tagged microsphere and a nucleus are encapsulated in a droplet, and the tag sequence carried by the microsphere can pair and bind to the cDNA capture adapter, and under the action of a polymerase, second-strand synthesis is completed, so that the cDNA acquires the tag sequence. The aqueous phase samples are collected by demulsification, the cDNA is purified therefrom, and cDNA enrichment and sequencing library construction are carried out by PCR, followed by high-throughput sequencing and analysis.

[0004] However, due to the relatively large number of centrifugation steps in snRandom-seq, the recovery efficiency of the nuclei by centrifugation is unstable, and the adhesion loss between the nuclei and the centrifuge tube and the pipette tip is relatively large, resulting in a relatively high requirement for the initial amount of nuclei; the adhesion problem between the nuclei is relatively severe, resulting in more cross-diffusion signals between different clusters, which affects the accuracy of subsequent analysis. Meanwhile, the nuclei of different types of cells in the tissue vary in size, and during multiple centrifugation processes, the nuclei are

repeatedly screened by size due to the centrifugation speed, resulting in an abnormal cell type distribution. There is a need to find a better method to achieve high-throughput single-cell nucleus transcriptome sequencing.

SUMMARY OF THE INVENTION:

[0005] Based on the above needs, the present invention provides a method for treating a tissue sample and a method for high-throughput transcriptome sequencing of a single-cell subcellular structure. In the present invention, after an optimized permeabilization treatment is performed on the tissue sample, in situ RNA labeling and capture adapter addition are carried out in the tissue, and then subcellular structures are dissociated from the tissue for single-cell subcellular structure partitioning, thereby achieving high-throughput single-cell transcriptome sequencing.

[0006] The present invention first provides a method for treating a tissue sample to obtain subcellular structures, which comprises the following steps:

[0007] S1-1: subjecting the tissue sample to fixation and permeabilization treatment;

[0008] S1-3: labeling RNA of the tissue sample, particularly by immersing the tissue sample in a labeling reagent, wherein the labeling reagent comprises a nucleotide chain, a tool enzyme, and a reaction buffer; through the binding of the nucleotide chain to the fragment of the RNA, the nucleotide chain binds to the chain of the RNA, and then a polymerization reaction or a ligation reaction occurs, causing the nucleotide chain to carry the information of the RNA and a capture adapter;

[0009] preferably, the labeling and adapter addition of the RNA of the tissue sample are achieved by reverse transcription; particularly, labeling RNA is performed by adding a reverse transcriptase, a nucleotide chain, and a reaction buffer, and capture adapter addition is performed by adding a terminal transferase and a deoxyribonucleoside triphosphate;

[0010] S1-4: adding a dissociation buffer to the tissue sample, then subjecting the tissue section to dispersion treatment, and performing centrifugation to remove a supernatant so as to obtain a subcellular structure.

[0011] In S1-1, the order of performing fixation and permeabilization on the tissue sample is not limited, and the fixation is to make the nucleic acid molecules in the tissue cross-link in the tissue. The permeabilization renders the interstitial space of the tissue and the biological membrane permeable, allowing proteins and nucleic acid molecules to pass through.

[0012] Particularly, the specific operation method of S1-1 is: for example, for a paraffin sample, performing deparaffinization, hydration, and permeabilization treatment; for a fixed

sample, performing permeabilization treatment; for a fresh or cryopreserved tissue sample, performing fixation and permeabilization treatment, preferably, the permeabilization treatment is a treatment using a surfactant, an enzyme, an acid, a high temperature, or a combination thereof, and a fixative used in the fixation is a single fixative or a mixed fixative; preferably, the single fixative comprises but is not limited to paraformaldehyde, formaldehyde, formalin, methanol, acetone, ethanol, acetic acid, picric acid, chromic acid, potassium dichromate, and mercuric chloride, and the mixed fixative comprises but is not limited to acetic acid-alcohol mixture, formalin-acetic acid-alcohol solution, and Bouin's fixative;

[0013] more preferably, the tissue sample after fixation is immersed in a permeabilization reagent for treatment, or is treated at a temperature of 70°C-90°C.

[0014] In a specific embodiment, the permeabilization treatment employs a permeabilization reagent which is a protease, preferably a 0.1 M hydrochloric acid solution of 1 mg/ml pepsin; the treatment is carried out at 35-39°C (36-38°C, particularly 37°C) for 5-30 minutes, for example, 8-12 minutes, particularly 10 minutes;

[0015] preferably, the protease may be one or more of pepsin, trypsin, neutral protease, papain, or collagenase.

[0016] In a specific embodiment, the method further comprises step S-2 between step S-1 and step S-3: blocking a single-stranded DNA in the tissue sample using a blocking reagent, wherein a method for the blocking comprises using a polymerase to fill in the single-stranded DNA to form a double-stranded DNA, or using a DNase to degrade the DNA single strand, and more preferably, performing a blocking reaction by adding a DNA polymerase, a blocking primer, dNTPs, and a reaction buffer to achieve the blocking;

[0017] preferably, the blocking reagent comprises 1 to 10 U/μl DNA polymerase, 5 to 50 mM blocking primer, 1 to 10 mM dNTPs, and 1X DNA polymerase buffer; the blocking reaction is carried out at 35-39°C (36-38°C, particularly 37°C) for 20 to 45 minutes.

[0018] In a specific embodiment, in S1-3, the nucleotide chain is a polyT sequence, a target gene sequence, a random sequence, or a combination thereof, wherein the random sequence can be composed of 2 bases, 3 bases, or 4 bases; the tool enzyme can be a reverse transcriptase, a polymerase, a ligase, a transposase, a terminal transferase, or a combination thereof.

[0019] In a specific embodiment, S1-3 is performed by incubation at 8-60°C (36-38°C, particularly 37°C) for 1-8 hours, preferably 3-5 hours, particularly 4 hours.

[0020] In a specific embodiment, the dissociation buffer in S1-4 is a surfactant, a salt ion solution, a dissociation enzyme, or a mixture thereof, and the dissociation enzyme is

selected from proteinase K, collagenase, neutral protease, trypsin, elastase, hyaluronidase, and papain; the salt ion solution is selected from PBS, HEPES, TRIS, or SSC buffer; the surfactant is selected from NP-40, IGEPAL® CA-630, TritonX-100, Tween-20, Tween-80, CHAPS detergent, Brij-58, and octyl thioglucoside (OTG);

[0021] preferably, the dissociation buffer comprises 0.5%-2% NP-40 ionic surfactant, 1-5 mM $MgCl_2$ in 2X SSC buffer, and 0.1-15 mg/ml proteinase K;

[0022] in a preferred embodiment, in S1-4, the dispersion treatment of the tissue sample is to disrupt the tissue by mechanical action to dissociate the subcellular structure; preferably, the tissue is disrupted by homogenization, more specifically, the homogenization is performed by adding the tissue into a Dounce homogenizer for homogenization, and homogenizing for 10 to 30 times;

[0023] in a preferred embodiment, the subcellular structure isolated in S1-4 is a nucleus.

[0024] Optionally, the method further comprises a step of purifying the obtained subcellular structure, preferably washing with a buffer and then filtering with a 10-40 μm cell strainer to remove tissue debris; further preferably, in each of steps S1-1 to S1-4, washing with a buffer is performed 2-5 times, preferably 3 times, before proceeding to the next step; the buffer is selected from PBS, HEPES, TRIS, or SSC buffer;

[0025] in some specific embodiments, the tissue sample is in a form of a tissue section, particularly, the tissue section has a thickness of 5-100 μm, preferably 10-60 μm.

[0026] The present invention further provides a method for high-throughput transcriptome sequencing of a single-cell subcellular structure, which comprises a step of performing high-throughput transcriptome sequencing of a single-cell subcellular structure using the subcellular structure obtained by the method.

[0027] In a specific embodiment, a microfluidic droplet or a microwell plate system is used to perform partitioning of subcellular structures, and when a microfluidic droplet system is used to perform partitioning of the subcellular structures, the specific steps thereof are as follows:

[0028] S2-1 connecting a sample of the subcellular structures, an extension reaction reagent, encoded microspheres, and an oil phase to the corresponding liquid inlets of a microfluidic chip, respectively, to form a water-in-oil single droplet containing a single-cell subcellular structure, a single encoded microsphere, and the extension reaction reagent, and collecting the single droplets to form cell partitions in which a single chamber comprises a single-cell subcellular structure;

[0029] S2-2 dispensing the collected single droplets into different tubes and then performing an extension reaction to synthesize a barcode-labeled cDNA second strand in the single droplets;

[0030] S2-3 after the extension reaction is completed, disrupting the single droplets, purifying and extracting the cDNA from the tubes, and performing PCR amplification;

[0031] S2-4 subjecting the amplified cDNA to end repair and A-tailing by a TA cloning and ligation adapter library construction method, and

[0032] ligating an adapter using a library construction kit;

[0033] S2-5 subjecting the constructed library to high-throughput sequencing on an Illumina sequencing platform.

[0034] In more specific embodiments, in S2-1, the connection is established to the corresponding liquid inlets of the microfluidic chip respectively via flexible tubing; in S2-3, a portion of the cDNA is taken as a template for a qPCR assay to detect the total cDNA content captured.

[0035] The present invention, during the preliminary sample treatment for single-cell subcellular structure sequencing, by subjecting the tissue to a preliminary permeabilization treatment, enhances the permeability of the tissue interstitium and biological membranes, and then, by hybridizing the nucleotide chain with the RNA in the tissue block, captures and labels the RNA in the cells. The operations on the tissue avoid the extensive centrifugation process during the treatment of a cellular subcellular structure. The subcellular structures in the tissue vary in size, and during multiple centrifugation processes, the subcellular structures are repeatedly screened by size due to the centrifugation speed, the centrifugation recovery efficiency varies among subcellular structures, and the adhesion loss of the subcellular structures in the centrifuge tube and the pipette tip during the operation is also relatively large, and the adhesion between the subcellular structures is also more prevalent, resulting in inconvenient operation and also affecting the final results.

[0036] When labeling the RNA in the cellular subcellular structures, multiple reagents are required, and before switching to a different reagent, a buffer needs to be used to wash away the previous reagent to reduce the interference of residual reagents on the next reaction. The pipette tip diameter used in conventional washing processes is about 0.36-1.5 mm, so the prior art requires the use of centrifugation to enrich the subcellular structures to prevent the subcellular structures from being inadvertently aspirated by the pipette during the washing process. In the RNA labeling stage for the cellular subcellular structures of the present invention, the subcellular structures still remain within the tissue, and the tissue has a size

exceeding the diameter of a conventional pipette tip, so the waste liquid can be directly aspirated using a pipette with a tip diameter of 0.36-1.5 mm and discarded, without the need to worry about the pipette aspirating the tissue block, thereby reducing the centrifugation operations during the washing process. In the present invention, the sample is dissociated into subcellular structures only after the RNA labeling is completed, and only 2-3 centrifugation and washing operations are required. Compared with the prior art, the number of centrifugation steps is reduced, the damage to the subcellular structures caused by the centrifugation process is reduced, the recovery efficiency of the subcellular structures is improved, the completeness of cell types is improved, and the aggregation of the subcellular structures caused by the centrifugation process is reduced.

[0037] Existing mainstream high-throughput single-cell transcriptome sequencing technologies: conventional microfluidic 10X Genomics, inDrop, Drop-seq; fixed-sample MATQ-DropSEQ (US patent, WO 2023/034913 A2) and snRandom-seq (Chinese patent CN 202210174619.9). Compared with MATQ-DropSEQ and snRandom-seq (fixed sample), the present invention has fewer centrifugation operation steps for the single-cell subcellular structures, reduces the damage to the subcellular structures caused by the centrifugation process, improves the recovery efficiency of the subcellular structures, improves the completeness of cell types, and reduces the aggregation of the subcellular structures caused by the centrifugation process; and the present invention operates on the tissue, can analyze fresh, cryopreserved, fixed, and FFPE samples, is suitable for multiple tissue types, and is convenient for automation.

BRIEF DESCRIPTION OF FIGURES:

[0038] FIG. 1 shows a schematic diagram of the experimental workflow of the present invention.

[0039] FIG. 2 shows a schematic diagram of the experimental workflow of snRandom-seq.

[0040] FIG. 3 shows a schematic diagram of in situ reverse transcription.

[0041] FIG. 4 shows a schematic diagram of in situ capture adapter addition.

[0042] FIG. 5 shows a schematic diagram of a microfluidic chip.

[0043] FIG. 6 shows a gene distribution map of mouse brain sample sequencing according to the present invention and snRandom-seq.

[0044] FIG. 7 shows the tSNE clustering of mouse brain samples according to the present invention and snRandom-seq.

[0045] FIG. 8 shows the comparison of cluster types of mouse brain sample sequencing according to the present invention and snRandom-seq.

[0046] FIG. 9 shows the results of analyzing stomach samples using different single-cell nucleus dissociation methods of the present invention.

[0047] FIG. 10 shows the results of treating cryopreserved mouse brain samples according to the present invention.

[0048] FIG. 11 shows the sequencing analysis of single-cell ribosome and mitochondria.

[0049] FIG. 12 shows the results of analyzing micro-amount of liver puncture FFPE samples in Example 6.

[0050] FIG. 13 shows the results of analyzing two kidney FFPE samples in parallel in a single experiment in Example 7.

[0051] FIG. 14 shows the results of analyzing breast FFPE samples by adding a capture adaptor using a template switching strategy in Example 8.

DETAILED DESCRIPTION OF EMBODIMENTS:

[0052] The present invention is further described below through specific embodiments, in order to better understand the present invention, but the embodiments do not constitute a limitation on the present invention.

[0053] **Example 1: Treatment of FFPE tissue sample and obtaining nuclei**

[0054] A mouse brain FFPE sample was prepared, and 50 um tissue sections were cut using a microtome; the sections were divided into two portions, with one section each. The tissue sections were transferred into a 1.5 ml centrifuge tube using forceps, and 1 ml of environmentally friendly deparaffinization solution (Solarbio, YA0031) was added. The tube was placed in a thermostatic mixer (Yooning Instrument, HH-100) at room temperature and 500 rpm for 10 minutes. The deparaffinization solution was directly aspirated using a pipette (tip diameter 0.75 mm, biosharp 1 ml pipette tip) and discarded, fresh deparaffinization solution was added, and this was repeated 1-2 times until deparaffinization was completed. After deparaffinization was completed, the deparaffinization solution was directly aspirated using a pipette and discarded, and gradient concentrations of ethanol (100%, 95%, 80%, 70%, 50%, 30%) were sequentially added for rehydration, and the waste liquid was directly aspirated using a pipette and discarded during the dehydration process.

[0055] The operation of the present invention was as follows (FIG. 1): After rehydration, the tissue sections were immersed in a permeabilization reagent (1 mg/ml pepsin (Sigma), 0.1 M hydrochloric acid) and placed at 37°C for 10 minutes. The tissue sections were washed 3

times with PBST, and during the subsequent tissue section operation process, the waste liquid was directly aspirated using a pipette and discarded. A blocking reagent (containing DNA polymerase (1 to 10 U/μl), blocking primer GAGAATGTGAGTGAAGATGTATGGTGANNNNNNN (5 to 50 mM), dNTPs (1 to 10 mM), 1X polymerase reaction buffer, and RNase inhibitor) was added, and the mixture was reacted at 37°C for 20 to 45 minutes in a PCR instrument. After the blocking was completed, the tissue sections were washed three times with PBST. The tissue sections were immersed in a reverse transcription reagent (comprising a reverse transcriptase, a reverse transcription reaction buffer, dNTPs, and a reverse transcription primer GGAGTTGGAGTGAGTGGATGAGTGATGNNNNNNN) to perform reverse transcription (FIG. 3). After the reaction was completed, the tissue sections were washed three times with PBST. A terminal transferase, dATP, and a reaction buffer were added to the sample, and the sample was incubated at 37°C for 30 minutes (FIG. 4). After the reaction was completed, the sample was washed three times with PBST. 1 ml of single-cell nucleus dissociation buffer was added to the tissue section, wherein the single-cell nucleus dissociation buffer was 2X SSC buffer containing 0.5 to 2% NP-40 ionic surfactant and $MgCl_2$ (1 to 5 mM). The mixture of the tissue section and the dissociation buffer was added into a Dounce homogenizer for homogenization, and homogenized 10 to 30 times, and left to stand on ice for 5 to 15 minutes. After homogenization, 100 μl of proteinase K (0.1 to 15 mg/ml) was added to the mixture, and the mixture was reacted at 25 to 50°C for 5 to 30 minutes. The nuclei were filtered using a 10-40 μm cell strainer to remove tissue debris. After centrifugation, the supernatant was aspirated and discarded, and centrifugal washing was performed three times with washing buffer. A portion of the nuclei was taken, stained with a nucleus-specific dye DAPI and observed and counted under a microscope.

[0056] As a control, the snRandom-seq method of the prior art was used (FIG. 2), and the operation was carried out as described in the literature High-throughput single nucleus total RNA sequencing of formalin-fixed paraffin-embedded tissues by snRandom-seq (Journal Nature Communications 2023), including tissue nuclear dissociation, blocking, in situ reverse transcription, capture adapter addition, and washing, followed by encapsulation in microfluidic droplets. The method specifically comprised: The rehydrated tissue section was immersed in 900 μl of single-cell nucleus dissociation buffer (1X PBS buffer, 0.1% Triton X-100, 1 U/μL RNase Inhibitor), added into a Dounce homogenizer for homogenization, and homogenized 30 times, and left to stand on ice for 10 minutes. After homogenization, 100 μl of proteinase K (10 mg/ml) was added to the mixture, reacted at 37°C

for 5 minutes, and the nuclei were filtered using a 40 μm cell strainer to remove tissue debris. The nuclei were centrifuged, the supernatant was aspirated and discarded, and the nuclei were centrifugally washed twice with PBST. A portion of the nuclei was taken, stained with a nucleus-specific dye DAPI and observed and counted under a microscope. 1 million nuclei were taken and centrifuged, and the nuclei were suspended in 25.5 μl of PBST. A blocking reaction reagent (2 μl of T4 DNA polymerase (1 to 10 U/μl), 5 μl of blocking primer (10 uM), 5 μl of dNTPs (100 mM), 10 μl of 5X polymerase reaction buffer, and 2.5 μl of RNase inhibitor) was added. The mixture was reacted at 37°C for 30 minutes in a PCR instrument. After the blocking was completed, the nuclei were centrifugally washed 3 times with PBST, and the supernatant was aspirated and discarded. The nuclei were collected and suspended in 22.5 μl of PBST. A reverse transcription reagent (comprising 2.5 μl of reverse transcriptase, 10 μl of 5X reverse transcription reaction buffer, 2.5 μl of dNTPs, 10 μl of random sequence reverse transcription primer, and 2.5 μl of RNase inhibitor) was added for reverse transcription. The reaction temperature was 12 cycles (8-42°C), followed by 42°C for 30 minutes. After reverse transcription was completed, the nuclei were collected, and then washed 3 times with PBST by centrifugation, with the supernatant aspirated and discarded after each centrifugation, to remove excess primers. The nuclei were suspended in 39 μl of PBST, and 0.5 μl of terminal transferase, 0.5 μl of dATP, and 10 μl of terminal transferase reaction buffer were added to the sample, and the mixture was incubated at 37°C for 30 minutes. After the reaction was completed, the steps of washing with PBST, centrifuging, and aspirating and discarding the supernatant were repeated three times. The nuclei were filtered using a 10 um cell strainer to remove aggregates. A portion of the nuclei was taken, stained with a nucleus-specific dye DAPI and observed and counted under a microscope for later use.

[0057] Example 2: Single-cell nucleus partitioning and high-throughput sequencing

[0058] The nuclei prepared by the method of the present invention and those prepared by the snRandom-seq method were used for microfluidic droplet production under the same conditions.

[0059] 50 μl of the single-cell nucleus sample and 50 μl of OptiPrep (Sigma) were mixed to prepare a nucleus suspension, and 100 μl of an extension reaction reagent (including 7.5 μl of DNA polymerase, 7.5 μl of RNase H (NEB), 7.5 μl of USER (NEB), 7.5 μl of dNTPs, 20 μl of 10x polymerase reaction buffer, and 50 μl of ddH_2O) was prepared. 100 μl of the cell suspension, 100 μl of the extension reaction reagent, 50 μl of encoded microspheres, and 200 μl of 7500 oil were separately added into syringes and connected to the corresponding liquid

inlets of the microfluidic chip (FIG. 5) respectively via flexible tubing. Appropriate flow rates were set to form water-in-oil single droplets each comprising a single nucleus, a single encoded microsphere, and the extension reaction reagent, and the single droplets were collected to form cell partitions in which a single chamber comprised a single cell.

[0060] The collected single droplets were dispensed into different 200 μl centrifuge tubes and placed in a PCR instrument for reaction (program: 37°C for 1 hour, 50°C for 30 minutes, 60°C for 30 minutes, 75°C for 20 minutes) to perform an extension reaction, wherein RNase H digested the RNA to release the cDNA from the nucleus, USER cleaved the tag sequence from the microsphere, and the free tag sequence was bound to the cDNA via the adapter sequence, and polymerized to form a barcode-labeled cDNA second strand. After the extension reaction was completed, PFO was added to disrupt the single droplets, and the cDNA in the tubes was purified and extracted by a magnetic bead method. A portion of the double-stranded cDNA was taken as a template for a qPCR assay, and the total cDNA content captured was evaluated by the CT value. The remaining cDNA was subjected to PCR amplification, and the amplified cDNA was subjected to end repair and A-tailing by a TA cloning and ligation adapter library construction method, and the adapters were ligated using a library construction kit. The constructed library was subjected to high-throughput sequencing on an Illumina sequencing platform.

[0061] The sequencing results showed a comparison between the snRandom-seq method and the method of the present invention for the detection of the same mouse brain FFPE sample. With the same amount of sequencing data (similar sequencing depth and cell count), based on the genome gtf annotation file, the numbers of reads mapped to different regions such as coding, intron, and intergenic regions were counted, and the numbers of detected genes were determined. The similarity of gene types was relatively high (FIG. 6) (refer to RSeQC: quality control of RNA-seq experiments), and the method of the present invention detected more coding RNA and had a lower number of intergenic reads, providing more effective gene information for downstream analysis. The data were subjected to cluster analysis (refer to the Seurat software official website description https://satijalab.org/seurat/), and the present invention showed cleaner clustering (FIG. 7). In snRandom-seq, due to the relatively large number of centrifugation steps, the adhesion problem between nuclei was relatively severe, resulting in more cross-diffusion signals between different clusters, which affected the accuracy of subsequent analysis. By statistically counting the numbers of cell types in FIG. 7, it can be found that the excessive number of centrifugation steps in snRandom-seq resulted in an uneven distribution of cell types, with smaller numbers of

multiple cell types (FIG. 8). The cell types obtained by the present invention were more uniform and diverse.

[0062] Example 3: Comparison of single-cell nucleus dissociation methods

[0063] Using the protocol of the present invention described in Example 1, deparaffinization, rehydration, permeabilization, blocking, reverse transcription, and adapter addition reactions were performed on a stomach FFPE sample. Then the tissue piece was divided into two portions. To one portion, a surfactant was added as the nucleus dissociation buffer, and the tissue was disrupted using a homogenizer. The nuclei were filtered through a 10-40 μm cell strainer to remove tissue debris, and were washed 3 times with PBST by centrifugation to obtain single nuclei, which were labeled as the mechanical dissociation group; To the second portion, a nucleus dissociation buffer containing a surfactant and proteinase K (0.1 to 15 mg/ml) was added, reacted at 25 to 50°C for 5 to 30 minutes. A pipette was used to pipette to assist the dissociation. The nuclei were filtered using a 10-40 μm cell strainer to remove tissue debris, followed by centrifugal washing 3 times with PBST to obtain single nuclei, which were labeled as the enzymatic dissociation group. The nuclei of the two groups were subjected to single-cell nucleus transcriptome analysis as described in Example 2. The results were as shown in FIG. 9. Both dissociation methods were able to obtain relatively good single-cell clustering (FIG. 9, panel a), and the genes detected in the two groups were highly consistent (FIG. 9, panel b), and the cell type distributions were also relatively consistent (FIG. 9, panel c).

[0064] Example 4: Treatment of cryopreserved samples and analysis of single-cell nucleus

[0065] A cryopreserved mouse brain tissue was prepared, and 30 um tissue sections were cut using a microtome. The tissue was fixed with 4% PFA for 15 min, and the tissue sections were washed 3 times with PBST, and the waste liquid was directly aspirated and discarded using a pipette. The tissue sections were immersed in a permeabilization reagent (containing 0.1% Triton-100, PBS) and placed at 4°C for 10 minutes. The tissue sections were washed 3 times with PBST, and the waste liquid was directly aspirated using a pipette and discarded. The tissue sections were immersed in a reverse transcription reagent (comprising a reverse transcriptase, a reverse transcription reaction buffer, dNTPs, and a reverse transcription primer) to perform reverse transcription. After the reaction was completed, the tissue sections were washed three times with PBST, and the waste liquid was directly aspirated using a pipette and discarded. A terminal transferase, dATP, and a reaction buffer were added to the sample, and the sample was incubated at 37°C for 30 minutes. After the reaction was

completed, the sample was washed three times with PBST, and the waste liquid was directly aspirated using a pipette and discarded. 1 ml of single-cell nucleus dissociation buffer was added to the tissue section, wherein the single-cell nucleus dissociation buffer was 2X SSC buffer containing 0.5 to 2% NP-40 ionic surfactant, MgCl2 (1 to 5 mM), and proteinase K (0.1 to 15 mg/ml). The mixture of the tissue section and the dissociation buffer was added into a Dounce homogenizer for homogenization, and homogenized 10 to 30 times, and the mixture was reacted at 25 to 50°C for 5 to 30 minutes. The nuclei were filtered using a 10-40 μm cell strainer to remove tissue debris. After centrifugation, the supernatant was aspirated and discarded, and centrifugal washing was performed 3 times with PBST. The nuclei were subjected to single-cell nucleus transcriptome analysis as described in Example 2. The results were as shown in FIG. 10. Clear single-cell clustering can be obtained using the cryopreserved mouse brain tissue (FIG. 10).

[0066] Example 5: Sequencing analysis of single-cell ribosome and mitochondria

[0067] Using the protocol of the present invention described in Example 1, deparaffinization, rehydration, permeabilization, blocking, reverse transcription, and adapter addition reactions were performed on a breast FFPE sample. A dissociation buffer containing collagenase was added to the tissue section, the tissue was disrupted using a homogenizer, and incubated at 37°C for 30 minutes. The nuclei were filtered using a 10-40 μm cell strainer to remove tissue debris, and centrifugally washed 3 times with PBST to obtain a single-cell nucleus mitochondria-ribosome complex, which was subjected to microfluidic partitioning and sequencing analysis as described in Example 2. The results were as shown in FIG. 11. A large number of ribosomes and mitochondria were retained in the isolated subcellular structures, and the single-cell nucleus mitochondria-ribosome complex exhibited relatively good clustering, wherein about 59.8% of the signals came from ribosomes, and the mitochondrial proportion among the coding RNA signals was also relatively high.

[0068] Example 6: Single-cell nucleus transcriptome analysis of puncture samples

[0069] 2-3 rolls of liver puncture FFPE samples were taken, and deparaffinization, rehydration, permeabilization, blocking, reverse transcription, and adapter addition reactions were performed using the method described in Example 1. During the operation on the tissue section, since the tissue section was relatively small, a tabletop centrifuge was used for brief centrifugation to collect the tissue before aspirating and discarding the liquid. Then, using the method described in Example 1, nuclear dissociation and washing were performed on the puncture sample, and the nuclei were collected and counted. Microfluidic partitioning and sequencing analysis were performed as described in Example 2. The sequencing data were as

shown in Table 1 below, and the results were as shown in FIG. 12. For micro-amount of puncture samples, the protocol of the present invention was also able to effectively perform single-cell nucleus transcriptome analysis, and clear single-cell clustering can be obtained using the liver puncture sample.

[0070] Table 1

Raw data (G)	Raw reads (M)	Aligned reads	Number of cells	Reads: UMI	Median reads	Median UMI	Median genes	Median mitochondrial proportion (%)
10.7	35.7	31.4	8363	1.1	778	672	494	6.47

[0071] **Example 7: Parallel single-cell nucleus transcriptome analysis of FFPE samples**

[0072] 2 human kidney FFPE section samples, one roll each, were taken, and deparaffinization, rehydration, permeabilization, blocking, reverse transcription, and adapter addition reactions were performed using the method described in Example 1. The reverse transcription primers used for the 2 samples carried different tag sequences (reverse transcription primer structure: PCR adapter + tag sequence + RNA binding sequence GAGAATGTGAGTGAAGATGTATGGTGA + GAAGGAAT + NNNNNNN; GAGAATGTGAGTGAAGATGTATGGTGA + GATGAATG + NNNNNNN), and after reverse transcription, the 2 samples were mixed together for the subsequent adapter addition, nuclear dissociation, washing, and counting operations. Microfluidic partitioning and sequencing analysis were performed as described in Example 2. The sequencing data were as shown in Table 2. In the sequencing data, the raw data could be split based on the tag sequences on the reverse transcription primers to obtain the data of the two samples. The results were as shown in FIG. 13. Both samples were effectively subjected to single-cell nucleus transcriptome analysis and clear single-cell clustering could be obtained.

[0073] Table 2

Raw data (G)	Raw reads (M)	Sample	Aligned reads	Number of cells	Reads: UMI	Median reads	Median UMI	Median genes	Median mitochondrial proportion (%)
23.3	77.6	NO.1	19.2	9209	1	311	304	228	14.77
		NO.2	35.6	4575	1	588	574	396	11.61

[0074] **Example 8: Single-cell nucleus analysis by adding capture adapters through template switching**

[0075] 1 roll of a breast FFPE sample was taken, and deparaffinization and rehydration were performed using the method described in Example 1. Then, the sample was washed 3 times with 0.1 M HCl and once with TE (pH 9.0), resuspended in TE (pH 9.0), treated in a metal bath at 95°C for 30 minutes, slowly cooled to room temperature, and washed 3 times with 2X SSCT (room temperature). The tissue section was immersed in a reverse transcription reagent (comprising a reverse transcriptase, a reverse transcription reaction buffer, dNTPs, a reverse transcription primer, and a template switching primer) for reverse transcription (template switching primer structure: TTTTTTTTTTTTTTTTrGrG/iXNA_G/). After the reaction was completed, the tissue section was washed three times with PBST. 1 ml of single-cell nucleus dissociation buffer was added to the tissue section, wherein the single-cell nucleus dissociation buffer was 2X SSC buffer containing 0.5 to 2% NP-40 ionic surfactant and $MgCl_2$ (1 to 5 mM). The mixture of the tissue section and the dissociation buffer was added into a Dounce homogenizer for homogenization, and homogenized 10 to 30 times, and left to stand on ice for 5 to 15 minutes. After homogenization, 100 μl of proteinase K (0.1 to 15 mg/ml) was added to the mixture, and the mixture was reacted at 25 to 50°C for 5 to 30 minutes. The nuclei were filtered using a 10-40 μm cell strainer to remove tissue debris. After centrifugation, the supernatant was aspirated and discarded, and centrifugal washing was performed three times with washing buffer. A portion of the nuclei was taken, stained with a nucleus-specific dye DAPI and observed and counted under a microscope. Microfluidic partitioning and sequencing analysis were performed as described in Example 2. The sequencing data were as shown in Table 3 below. The results were as shown in FIG. 14. By template switching, rather than adding a polyA tail by terminal transferase, the capture adapter could also be effectively added at the end of the single-cell nucleus cDNA, achieving single-cell nucleus transcriptome analysis and obtaining clear single-cell clustering.

[0076] Table 3

Raw data (G)	Raw reads (M)	Aligned reads	Number of cells	Reads: UMI	Median reads	Median UMI	Median genes	Median mitochondrial proportion (%)
7.2	23.9	19.6	2830	1.6	690	435	355	9.63

Industrial applicability

[0077] The subcellular structure obtained by the present invention is highly conducive to high-throughput transcriptome sequencing of a single-cell subcellular structure, and thus has great practical value.

CLAIMS

1. A method for treating a tissue sample to obtain a single-cell subcellular structure, characterized in that the method comprises the following steps:

S1-1: subjecting the tissue sample to fixation and permeabilization treatment;

S1-3: labeling RNA of the tissue sample, particularly by immersing the tissue sample in a labeling reagent, wherein the labeling reagent comprises a nucleotide chain, a tool enzyme, and a reaction buffer; through the binding of the nucleotide chain to the fragment of the RNA, the nucleotide chain binds to the chain of the RNA, and then a polymerization reaction or a ligation reaction occurs, causing the nucleotide chain to carry the information of the RNA and a capture adapter;

preferably, the labeling of the RNA of the tissue sample is achieved by a reverse transcriptase, particularly by adding the reverse transcriptase, a nucleotide chain, and a reaction buffer to perform RNA reverse transcription to produce cDNA carrying the information label of the RNA, and adding terminal transferase and a deoxyribonucleoside triphosphate to perform capture adapter addition to the cDNA, or template switching to add a capture adapter to the end of the cellular cDNA;

S1-4: adding a dissociation buffer to a tissue section, then subjecting the tissue section to dispersion treatment, and performing centrifugation to remove a supernatant so as to obtain a subcellular structure; the subcellular structure is a nucleus, an organelle (such as mitochondria, ribosome, lysosome, endoplasmic reticulum, Golgi apparatus), a cytoplasmic condensate, a plastid, a lysosome, a physical structure secreted by a cell (such as an extracellular vesicle), or a combination thereof; preferably, the subcellular structure is a nucleus.

2. The method according to claim 1, characterized in that an original sample of the tissue sample is a paraffin-embedded sample, a cryopreserved sample, a fresh sample, a refrigerated sample, a fixative-preserved sample, a sample preservation solution-preserved sample, or a puncture sample;

the specific operation method of S1-1 is: for a paraffin-embedded sample, performing deparaffinization, hydration, and permeabilization treatment; for a fixed sample, performing permeabilization treatment; for a fresh or cryopreserved tissue sample, performing fixation and permeabilization treatment;

preferably, the permeabilization treatment is a treatment using a surfactant, an enzyme, an acid, a high temperature, or a combination thereof, and a fixative used in the fixation is a single fixative or a mixed fixative; preferably, the single fixative comprises but is not limited to paraformaldehyde, formaldehyde, formalin, methanol, acetone, ethanol, acetic acid, picric acid, chromic acid, potassium dichromate, and mercuric chloride, and the mixed fixative comprises but is not limited to acetic acid-alcohol mixture, formalin-acetic acid-alcohol solution, and Bouin's fixative;

more preferably, the permeabilization treatment is performed by immersing the tissue sample in a permeabilization reagent, or by treatment at a temperature of 70°C-90°C.

3. The method according to claim 2, characterized in that the permeabilization treatment in S1-1 employs a protease treatment, preferably a 0.1 M hydrochloric acid solution of 1 mg/ml pepsin; the treatment is carried out at 35-39°C (36-38°C, particularly 37°C) for 5-30 minutes, for example, 8-12 minutes, particularly 10 minutes;

preferably, the protease may be one or more of pepsin, trypsin, neutral protease, papain, or collagenase.

4. The method according to claim 1, characterized in that the method further comprises step S-2 between step S-1 and step S-3: blocking a single-stranded DNA in the tissue sample using a blocking reagent, wherein a method for the blocking comprises using a polymerase to fill in the single-stranded DNA to form a double-stranded DNA, or using a DNase to degrade the DNA single strand, and more preferably, performing a blocking reaction by adding a DNA polymerase, a blocking primer, dNTPs, and a reaction buffer to achieve the blocking;

preferably, the blocking reagent comprises 1 to 10 U/μl DNA polymerase, 5 to 50 mM blocking primer, 1 to 10 mM dNTPs, and 1X DNA polymerase reaction buffer; the blocking reaction is carried out at 35-39°C (36-38°C, particularly 37°C) for 20 to 45 minutes.

5. The method according to claim 1, characterized in that in S1-3, the nucleotide chain is a polyT sequence, a target gene sequence, a random sequence, or a combination thereof, wherein the random sequence can be composed of 2 bases, 3 bases, or 4 bases; the tool enzyme can be a reverse transcriptase, a polymerase, a ligase, a transposase, a terminal transferase, or a combination thereof.

6. The method according to claim 1, characterized in that the labeling RNA in S1-3 is performed by incubation at 8-60°C (36-38°C, particularly 37°C) for 1-8 hours, preferably 3-5 hours, particularly 4 hours.

7. The method according to claim 1, characterized in that the dissociation buffer in S1-4 is a surfactant, a salt ion solution, a dissociation enzyme, or a mixture thereof, and the dissociation enzyme is selected from proteinase K, collagenase, neutral protease, trypsin, elastase, hyaluronidase, and papain; the salt ion solution is selected from PBS, HEPES, TRIS, or SSC buffer; the surfactant is selected from NP-40, IGEPAL® CA-630, TritonX-100, Tween-20, Tween-80, CHAPS detergent, Brij-58, and octyl thioglucoside (OTG);

preferably, the dissociation buffer comprises 0.5%-2% NP-40 ionic surfactant, 1-5 mM $MgCl_2$ in 2X SSC buffer, and 0.1-15 mg/ml proteinase K.

8. The method according to claim 1, characterized in that in S1-4, the dispersion treatment of the tissue sample is to disrupt the tissue by mechanical action to dissociate the subcellular structure; preferably, the tissue is disrupted by homogenization, and tissue fragments are reacted at 25-50°C for 5-30 minutes.

9. The method according to claim 8, characterized in that the method further comprises a step of purifying the obtained subcellular structure, preferably washing with a buffer and then filtering with a 10-40 μm cell strainer to remove tissue debris; further preferably, in each of steps S1-1 to S1-4, washing with a buffer is performed 2-5 times, preferably 3 times, before proceeding to the next step; the buffer is selected from PBS, HEPES, TRIS, or SSC buffer.

10. The method according to any one of claims 1 to 9, characterized in that the tissue sample is in a form of a tissue section, particularly, the tissue section has a thickness of 5-100 μm, preferably 10-60 μm;

optionally, the tissue sample is a mixed sample of two or more samples labeled with tag sequences on different reverse transcription primers.

11. A method for high-throughput transcriptome sequencing of a single-cell subcellular structure, characterized in that the method comprises a step of performing high-

throughput single-cell subcellular structure sequencing using the subcellular structures obtained by the method according to any one of claims 1 to 10.

12. The method according to claim 11, characterized in that a microfluidic droplet or a microwell plate system is used to perform partitioning of subcellular structures of a single-cell;

preferably, steps of performing partitioning of subcellular structures of a single-cell using a microfluidic droplet system are as follows:

S2-1 connecting a sample of the subcellular structures, an extension reaction reagent, encoded microspheres, and an oil phase to the corresponding liquid inlets of a microfluidic chip, respectively, to form a water-in-oil single droplet containing a single-cell subcellular structure, a single encoded microsphere, and the extension reaction reagent, and collecting the single droplets to form partitions in which a single chamber comprises a single-cell subcellular structure;

S2-2 dispensing the collected single droplets into different tubes and then performing an extension reaction to synthesize a barcode-labeled cDNA second strand in the single droplets;

S2-3 after the extension reaction is completed, disrupting the single droplets, purifying and extracting the cDNA from the tubes, and performing PCR amplification;

S2-4 subjecting the amplified cDNA to end repair and A-tailing by a TA cloning and ligation adapter library construction method, and ligating an adapter using a library construction kit;

S2-5 subjecting the constructed library to high-throughput sequencing on an Illumina sequencing platform.