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NET LIFE SCIENCE

METHODS IN BIOLOGY

FOR CSIR NET LIFESCIENCE

MODULE 13

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A1. ISOLATION AND PURIFICATION OF RNA AND DNA

Before beginning any genetic engineering work, it is necessary to obtain a relatively pure DNA, which is then cut and ligated to genome. The basic procedure requires the releasing of the DNA from the cells and purification of the DNA to be used in the experiments.

Isolation and purification of DNA mainly include four steps:

1. Cell Lysis
2. Enzymatic treatment
3. Purification of DNA/RNA
4. Quantification of DNA/RNA

1. Cell lysis

Although there are many different methods for purifying nucleic acids, they have a number of basic features in common. Firstly we need the starting material. This could be a culture of bacterial or eukaryotic cells, which would simply need to be separated from growth medium (for example centrifugation), or a more complex tissue sample, which first needs to be homogenized so that individual cells can be lysed. Wherever possible, the material should be freshly harvested or frozen until ready to use, to avoid degradation by enzymes present in cell extracts.

The cells that need to be lysed to release their components. The nature of the treatment will vary widely according to the cell type. Bacterial cells have walls that have to be broken before the cell contents to be release. This is usually accomplished by using lysozyme, often in conjugation with EDTA and a detergent such as SDS. EDTA eliminates divalent cations and thus destabilized the outer membrane in bacteria such as *E. coli*, and also inhibits DNases that would otherwise tend to degrade the DNA, while the detergents will solubilize the membrane lipids.

Plant and fungal cells have cell walls that are different from those in bacteria, and require alternative treatments, either mechanical or enzymatic (Cellulase, chitinase), while animal cells (which lack a cell wall) can usually be lysed by more gentle treatment with mild detergents(such as CTAB).

After breaking up cell walls, and plasma membrane, we find ourselves with a mixture of material and intracellular components which have now been release-a complex solution of DNA, RNA, proteins, lipids and carbohydrates. Note that the sudden lysis of the cell will usually result in fragmentation of chromosomal DNA. In particular, the bacterial chromosome, which is usually circular in its native state, will be broken into linear fragments. Where it is necessary to obtain a large (even intact) chromosomal DNA, more gentle lysis conditions are necessary. Bacterial plasmids, however, are readily obtained in their native circular state by standard lysis conditions. The next step in the procedure is to separate the desired nucleic acid from other components.

2. Enzymatic treatment:

Removal of RNA from a DNA preparation is easily achieved by treatment with ribonuclease (RNase A). Since RNase is a very heat stable enzyme, it is easy to ensure that it is free of all traces of deoxyribonucleases (DNase) that would otherwise degrade your DNA, simply by heating the enzyme before use. Protein contamination can be removed by digestion with a proteolytic enzyme such as proteinase K.

These treatments are applied if necessary in different nucleic acid purification protocols. However, in some protocols they are omitted, either because the contamination is unimportant for a specific purpose, or because the contamination will be removed anyway through subsequent steps, as described below.

3. Purification of nucleic acids:

3.1 Phenol: chloroform extraction: Removal of proteins is particularly important as the cell contains a number of enzymes that will degrade nucleic acids, as well as other proteins that will interfere with subsequent procedures by binding to the nucleic acids. A classical, and still frequently employed way of removing proteins is by extraction with liquefied phenol, or preferably a mixture of phenol and chloroform. Phenol and chloroform are immiscible with water, and so you will get two layers (phases) when added to cell extract. When the mixture is vigorously agitated, the proteins will be denatured and precipitated at the interphase (Figure 1).

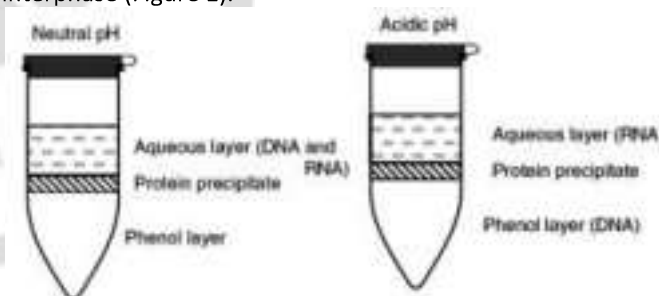


Figure 1: Phenol extraction

If you are using phenol that has been equilibrated with a neutral or alkaline buffer, the nucleic acids (DNA and RNA) will remain in the aqueous layer. On the other hand, if you carryout the extraction with acidic phenol, DNA will partition into the organic phase, allowing you to recover RNA from the aqueous phase. Phenol is naturally acidic, so equilibrate with water or use of an acidic buffer will produce the appropriate conditions

Phenol extraction is also useful in subsequent stages of manipulation when it is necessary to ensure that all traces of enzymes have been removed before proceeding into next step.

Stage 3. Sequencing the peptides—Once the peptide fragments are generated, we can start the sequencing of each polypeptide chain. It has following steps:

A. Identifying the N-terminal residue: The N-terminal amino acid analysis is a 3 steps process.

1. Derivatization of terminal amino acid—The chemical reaction is performed to labeled terminal amino group with compounds such as sanger reagent 1-fluoro-2,4-dinitrobenzene (DFNB) and dansyl chloride. In most of the case these reagents also label free amino group present on basic amino acids such as lysine and arginine. In a reaction mechanism given in figure 12, dinitrofluorobenzene reacts with the free amine group to form **dinitrophenyl-amino acid** complex.

2. hydrolyse the protein—Acid hydrolysis of dinitrophenyl-amino acid complex leads to the breaking of peptide bond to release dinitrophenyl-amino acid complex in solution.

3. Separation and analysis of derivatized amino acids

A HPLC or TLC separation of complex and comparing with the standard amino acids.

B. Edman Degradation sequencing:

Edman Degradation sequencing methods is given in Figure 12 and it has following steps:

1. Similar to sanger reagent, phenylisothiocyanate reacts with the terminal amino group to form a cyclic phenylthiocarbamoyl derivative.
2. Under acidic condition, the terminal amino acid is cleaved from the main chain as thiazolinone derivative.
3. Thiazolinone derivative is extracted into the organic solvent and it forms phenylthiohydantoin-amino acid (PTH-amino acid) derivative in the presence of acid.
4. PTH-amino acid acid complex can be identify by HPLC or TLC and comparison with the standard amino acids.
5. Step 1-4 can be repeated again with the next amino acid residue in the peptide chain.



Figure 12: Steps in Edman Degradation sequencing methods for protein

C. C-terminal residues: Not many methods are developed for c-terminal amino acid analysis. The most common method is to treat the protein with a carboxypeptidase to release the c-terminal amino acid and test the solution in a time dependent manner.

Stage 4. Ordering the peptide fragments:

The usage of different protein cleavage reagent produces over-lapping amino acid stretches and these stretches can be used to put the whole sequence.

Stage 5. Locating disulfide bonds:

The protein cleavage by trypsin is performed with or without breaking di-sulphide linkage. Amino acid sequence analysis of the fragments will provide the site of disulphide bond. The presence of one disulphide will reduce two peptide fragments and will appear as one large peptide fragment. For example: A 30-residue peptide with sequence

AEKCEPGYRCEHFRWCSPPKCGGKCEAFCL

was treated with trypsin and the tryptic peptides were separated by HPLC. Four peaks A, B, C and D will be obtained.

Peptides corresponding to A, B, C and D are reduced (β -ME) and alkylated (Iodoacetate) selectively at cysteine residues. The sequences obtained from A, B, C and D after reduction and alkylation are:

A: AEK;

B: C(S-alkyl)EPGYR and WC(S-alkyl)SPPK;

C: C(S-alkyl)EHFR and C(S-alkyl)GGK;

D: C(S-alkyl)EAFCL.

On basis of above information it can be concluded that peak B, C and D each have two peptides which were linked by disulphide bonds with each other and we can represent it as follows



The information can also be obtained by using a **diagonal electrophoresis** technique to isolate the peptide sequences containing such bonds (Figure 13).

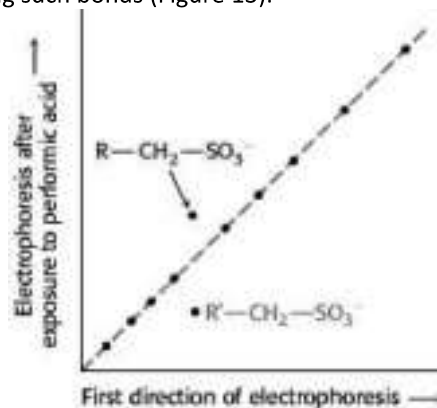


Figure 13: Diagonal electrophoresis technique for the peptide sequences containing disulfide bonds.

Above equation can be rewritten in terms of the molar absorption coefficients of the molecule for the left and right circularly polarized lights:

$$CD = (\epsilon_L - \epsilon_R) C l$$

where; ϵ_L , ϵ_R , C , and l represent the molar absorption coefficient for left circularly polarized light, molar absorption for right circularly polarized light, molar concentration of the molecule, and the path length of the cell, respectively.

$$CD = \Delta \epsilon C l$$

Differential absorption of the two circularly polarized lights results in elliptically polarized light and CD is historically represented in terms of ellipticity (θ) which is the angle whose tangent is the ratio of the minor to major axis of the ellipse (Figure 81).

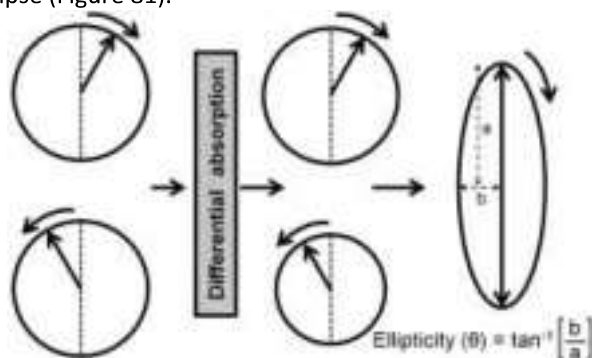


Figure 81: Differential absorption of left and right circular polarized light result in elliptically polarized light. Ellipticity is the arc tangent of the ratio of minor to major axis.

CD of proteins and peptides

Proteins are the linear polymers made up of 20 amino acids, 19 out of which (except glycine) are chiral. This chirality is also reflected in the secondary structures the proteins/peptides adopt. Far-UV CD spectra of proteins are typically recorded from 190 – 250 nm. Peptide bond is the major chromophore in this region and the relative orientations of the peptide bonds with respect to each other lead to characteristic CD signals thereby allowing identification of the secondary structural elements.

Peptide bond constitutes the major protein chromophore in the far-UV region. The absorbance in the far UV region is the manifestation of $n \rightarrow \pi^*$ transitions around 220 nm and $\pi \rightarrow \pi^*$ transitions below 210 nm. The orientation of the peptide bonds in the secondary structural elements greatly influences their CD signals. Let us have a look at the characteristic spectral features of the major secondary structural components present in proteins (Figure 82).

α -helix: CD spectrum of right-handed α -helix is characterized by two negative absorption bands of nearly same intensity centered around 222 nm (arises due to $n \rightarrow \pi^*$ transition) and 208 nm (a fraction of the $\pi \rightarrow \pi^*$ transition) and a relatively more intense positive band around 192 nm (a fraction of the $\pi \rightarrow \pi^*$ transition).

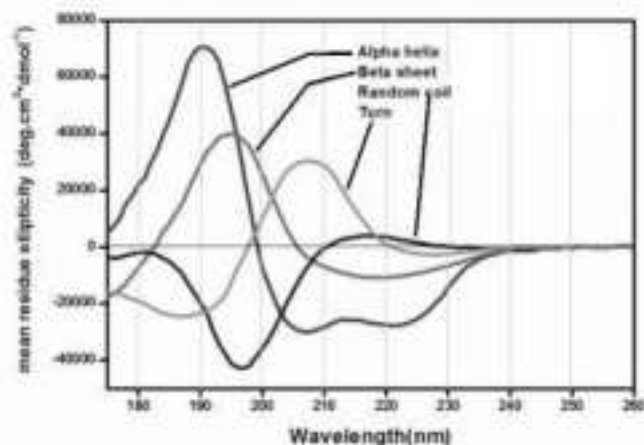


Figure 82: Far UV CD spectra of α -helix, β -sheets. Turn and unordered conformations.

β -sheet: β -sheets display a negative band centered around 216-218 nm (arises due to $n \rightarrow \pi^*$ transition) and a positive band of comparable intensity at ~ 195 nm (arises due to $\pi \rightarrow \pi^*$ transition).

β -turn: β -turn is a four residue protein motif that causes the polypeptide backbone to take a turn of approximately 180° . β -turns do not have a well defined spectral signature. A typical β -turn, however, shows a weak negative band around 225 nm (arises due to $n \rightarrow \pi^*$ transition), a strong positive band between 200 – 205 nm (arises due to $\pi \rightarrow \pi^*$ transition), and a strong negative band (arises due to $\pi \rightarrow \pi^*$ transition) between 180 – 190 nm.

Random coil: Random coil or the unordered conformation displays a weak positive band around 218 nm (arises due to $n \rightarrow \pi^*$ transition) and a strong negative band (arises due to $\pi \rightarrow \pi^*$ transition) below 200 nm.

Many different methods are available for analyzing the circular dichroism spectra of proteins. All these methods work on the assumption that the CD spectrum of the protein is a linear combination of the spectra of its secondary structural elements, plus noise. The ellipticity of the protein at any wavelength can therefore be represented by equation

$$\theta_\lambda = \sum f_i S_{i\lambda} + \text{noise}$$

Where θ_λ represents the ellipticity of the protein at wavelength, λ ; f_i , represents the fraction of the i th secondary structural element; and $S_{i\lambda}$ represents the ellipticity of the secondary structural element, S_i at wavelength, λ .

The methods that are in practice utilize the CD spectra derived from the proteins whose crystal structures have been determined as the reference. The initial attempts in deconvoluting the protein CD spectra utilized poly-L-lysine CD spectra as reference.

Following examples will make the distinction in between the two types of errors more clear. Suppose, the difference between two population means is zero, and if our test of significance indicates rejection of the null hypothesis, we commit type I error. On the other hand, if there is true difference between the two population means, our test of significance, suggests acceptance of null hypothesis then, we commit type II error. Table gives an idea about the type I and type II errors.

	Reject H_0	Accept H_0
H_0 is True	Type I error	Correct Decision
H_0 is False	Correct Decision	Type II error

Table can be expressed as follows

- (1) Rejects H_0 when it is true (Type I error) $= \alpha$
- (2) Accept H_0 when it is false (Type II error) $= \beta$
- (3) Accept H_0 when it is true (Correct decision)
- (4) Reject H_0 when it is false (Correct decision)

While testing hypothesis, we would like to minimize both the errors. However, due to the fixed sample size, it is not possible to control both the errors simultaneously. Researcher will be interested in deciding whether to accept or reject a null hypothesis. We always desire to minimize the chance of committing type I error (α) then the chance of committing type II error (β) will increase. In general, it is more risky to accept a false hypothesis (Type II error) than to reject a true hypothesis (Type I error).

Level of significance

The statistical tests fix the probability of committing type I error at a certain level, called the level of significance and minimize the chances of committing the type II error. The level of significance is thus, the probability of committing type I error and is usually denoted by α . The level of significance is usually chosen as 0.05 (5% level of significance) or 0.01 (1 % level of significance). 0.01 is used for high precision and $\alpha = 0.05$ for moderate precision. In most biometric test, the level of significance is generally fixed at 5%. This means that the probability of accepting a true hypothesis is 95%.

The problem before a researcher will be the choice of the level of significance. The choice of α is basically governed by the consequences of the two types of errors. These two types of errors can be easily understood with an example where a patient is given a drug to cure some disease. Type I error is when the drug has a positive effect, but it is considered that it has no effect or an adverse effect. Type II error, the drug has an adverse effect, but it is considered that it had positive effect. If the type I error is committed, the patient will be given another drug which may or may not be effective. On the other hand if type II error is committed the drug is continued in spite of an adverse effect. The patient is likely to develop some other complication, or may even die. This indicates that the type II error is more severe than the type I error.

Therefore, while drawing inferences about the null hypothesis, type II error be minimized even at a certain risk of type I error.

One and two-tailed tests of hypothesis

A sample statistics is used to test the hypothesis H_0 . In a test, the area under the probability curve is divided into two regions- the **acceptance region** and the **rejection or critical region**.

Significance of Difference in Means

Tests for significance of difference in means are discussed under two heads

- (1) Z- test for large samples.
- (2) T- test for small samples applied as unpaired (Two independent samples) and paired (Single sample correlated observations) test.

For application of either of the two tests 'Z' or 't'; standard error of mean i.e., a unit that measures chance variation has to be understood.

Standard Error of Mean:

The quantity calculated to represent a character of population is known as parameter whereas the calculated value presenting the character of the sample is known as statistics. The former is constant quality while the latter is variate. This property of statistics explains that when several samples are taken from population, their mean and standard deviation will vary from the parameter of the population. The difference of mean and standard deviation of samples from the parameter of the population is known as sample error.

Computation of standard error of mean $[SE \bar{X}]$

Standard error of means of samples drawn from a particular population could be calculated if means of large number of samples were known. To calculate the $SE \bar{X}$, find the mean (μ) of the sample means ($\bar{X}$) and then the differences of individual means from this grand mean ($\bar{X} - \mu$)

Standard deviation of a series of means

$$\text{or Standard error of mean} = \sqrt{\frac{\sum (\bar{X} - \mu)^2}{N - 1}}$$

$SE \bar{X}$ for individual series: In practice, usually only one large sample is drawn and its standard error of mean is calculated by the following formula.

$$SE \bar{X} = \frac{SD}{\sqrt{N}} \text{ or } \frac{\sigma}{\sqrt{N}}$$

Thus $SE \bar{X}$ is the SD of the sample divided by the square root of the number of observations in the sample.

2. ABSORBED DOSE UNITS:

a. Rad (Radiation Absorbed Dose) is the quantity of γ -rays or X-rays that can deposit 1 erg (or 10^{-5} J) of energy in 1 g of matter, including biological material. A unit of **absorbed dose** of ionizing radiation. (1 rad = 10 milligray)

b. Gray (Gy): A unit of **absorbed dose** of ionizing radiation. The dose is 1 Gray when the density of the total energy absorbed, in any medium from any type of ionizing radiation, is 1 Joule/kg.

The dose can be expected to vary from point to point within the irradiated object. (1 Gy = 100 Rad)

3. EXPOSURE UNITS:

Roentgen (R) is amount of γ -rays (X-rays) which produce 1.61×10^{12} ion pairs per gram or 2.08×10^9 ion pairs per cm^3 of dry air at STP or that quantity of X- or gamma-radiation such that the associated corpuscular emission per 0.001293 g of dry air (equals 1 cc at STP) produces, in air, ions carrying 1 esu of quantity of electricity of any one sign; alternatively, 258 microcoulombs/kg.

Equal doses of α, β, γ rays produce of tissue damage in descending order.

4. BIOLOGICALLY EFFECTIVE DOSE UNITS:

a. Relative Biological Effectiveness (RBE): is the ratio between the dose (Rad) of radiations under investigation and that of 200 KeV γ rays or X-rays, producing equivalent biological effects. Roentzen equivalent mammals (Rem) is measure of absorbed dose (Rad) & its effect on mammalian tissue (RBE).

$$\text{Rem} = \text{Rad} \times \text{RBE}$$

b. Sievert (Sv) is the SI unit for biologically equivalent radiation dose. Dose equivalent; dimensionally J/kg, but calculated as the product of the physical absorbed dose in Gy and the Relative Biological Effectiveness.

$$1 \text{ Sv} = 100 \text{ Rem}$$

$$1 \text{ Rem} = 10 \text{ Milli Sieverts}$$

$$\text{Sv} = \text{Gy} \times \text{Quality Factor}$$

c. Roentgen equivalent man (REM): That amount of ionizing radiation of any type which produces the same damage to humans as 1 roentgen of 250 kVp X-radiation.

d. Roentgen equivalent physical (REP): That amount of ionizing radiation of any type which results in the absorption of energy at the point in question in soft tissue to the extent of 93 ergs/g. It is approximately equal to 1 roentgen of 250 kVp X-radiation in soft tissue, or 0.93 rads, or 9.3 milligray.

5. GAS IONISATION CHAMBER:

A gas ionization counter detects and measures radiations on basis of their effect on gas molecules in producing ions pairs, which lower the potential difference between anode and cathode to generate a current pulse.

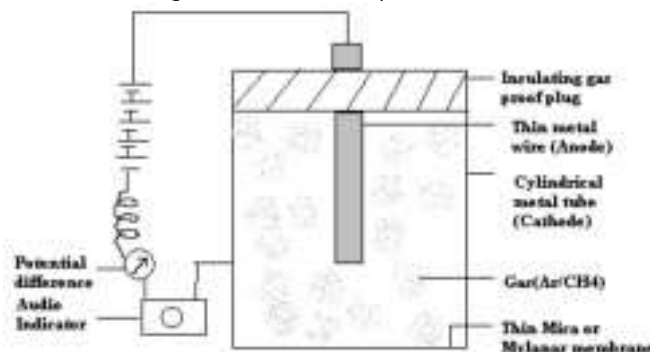


Figure 97: Geiger Muller Counter

Geiger Muller Counter:

This is gas ionization counter operated at very high voltage falling in Geiger regions (>200 V). Such high-applied voltage leads to high gas amplification due to extensive secondary ionization that the size of output voltage reaches 0.25-10 V and pulses can be counted even without the use of electronic pulse amplifier. In a Geiger-Muller counter (G-M tube), a high potential, above 1000 V, is applied to the central anode wire which is surrounded by a cylindrical cathode. The anodal axis wire is maintained so highly charged that the surrounding electric field stresses the gas inside the chamber almost to the breaking points of spark (Figure 97). The tube is filled with a gas mixture (argon + Methane/ethane) and sealed. Polyatomic gas (Ethanol/Ether) is mixed with gas mixture as easily dissociatable quenching agent.

A thin mica or glass window acts, as an entry area for incident radiation. An incident ionising particle initiates ion"pair production in the G-M tube. These ion-pairs collide with neighbouring gas molecules and ionise then. Such cascade collisions of ions produce an avalanche of ionisation, and the electrons upon reaching the anode produce photons which in turn produce ionisation throughout the tube. The amplification reaches $\sim 10^{10}$ counts/sec. Geiger counters are simple, rugged and easy to operate but sluggish. They are used for detection and monitoring of high-energy radiation and β -particles.

A pulse-height analyser can be incorporated into detectors, such as proportional counters, to make them operational in the restricted range of X-ray or γ -ray energies. A pulse-height analyser eliminates all the pulses of energies other than the preset pulse amplitudes. By this means energy-discrimination can be achieved.

While BOLD signal is the most common method employed for neuroscience studies in human subjects, the flexible nature of MR imaging provides means to sensitize the signal to other aspects of the blood supply. Alternative techniques employ arterial spin labeling (ASL) or weight the MRI signal by cerebral blood flow (CBF) and cerebral blood volume (CBV). The CBV method requires injection of a class of MRI contrast agents that are now in human clinical trials. Because this method has been shown to be far more sensitive than the BOLD technique in preclinical studies, it may potentially expand the role of fMRI in clinical applications. The CBF method provides more quantitative information than the BOLD signal, albeit at a significant loss of detection sensitivity.

Advantages of fMRI

It can noninvasively record brain signals (of humans and other animals) without risks of radiation inherent in other scanning methods, such as CT scans.

It can record on a spatial resolution in the region of 3-6 millimeters, but with relatively poor temporal resolution (on the order of seconds) compared with techniques such as EEG. However, this is mainly because of the phenomena being measured, not because of the technique. EEG measures electrical/neural activity while fMRI measures blood activity, which has a longer response. The MRI equipment used for fMRI can be used for high temporal resolution, if one measures different phenomena.

Disadvantages of fMRI

The BOLD signal is only an indirect measure of neural activity, and is therefore susceptible to influence by non-neural changes in the body.

BOLD signals are most strongly associated with the input to a given area rather than with the output. It is therefore possible (although unlikely) that a BOLD signal could be present in a given area even if there is no single unit activity.

Different brain areas have different hemodynamic responses, which would not be accurately reflected by the general linear model often used to filter fMRI time signals.

65. COMPUTED AXIAL TOMOGRAPHY (CAT)

Computed tomography (CT) is a medical imaging method employing tomography. Digital geometry processing is used to generate a three-dimensional image of the inside of an object from a large series of two-dimensional X-ray images taken around a single axis of rotation. The word "tomography" is derived from the Greek *tomos* (slice) and *graphein* (to write).

Computed tomography was originally known as the "EMI scan" as it was developed at a research branch of EMI, a company best known today for its music and recording business. It was later known as computed axial tomography (CAT or CT scan) and body section röntgenography.

CT produces a volume of data which can be manipulated, through a process known as windowing, in order to demonstrate various structures based on their ability to block the X-ray/Röntgen beam. Although historically (see below) the images generated were in the axial or transverse plane (orthogonal to the long axis of the body), modern scanners allow this volume of data to be reformatted in various planes or even as volumetric (3D) representations of structures.

Diagnostic use

Since its introduction in the 1970s, CT has become an important tool in medical imaging to supplement X-rays and medical ultrasonography. Although it is still quite expensive, it is the gold standard in the diagnosis of a large number of different disease entities. It has more recently begun to also be used for preventive medicine or screening for disease, for example CT colonography for patients with a high risk of colon cancer.

CT is excellent for detecting both acute and chronic changes in the lung parenchyma, that is, the internals of the lungs. It is particularly relevant here because normal two dimensional x-rays do not show such defects. A variety of different techniques are used depending on the suspected abnormality.

CT pulmonary angiogram (CTPA) is a medical diagnostic test used to diagnose pulmonary embolism (PE). It employs computed tomography to obtain an image of the pulmonary arteries.

It is a preferred choice of imaging in the diagnosis of PE due to its minimally invasive nature for the patient, whose only requirement for the scan is a cannula (usually a 20G).

With the advent of subsecond rotation combined with multi-slice CT (up to 64-slice), high resolution and high speed can be obtained at the same time, allowing excellent imaging of the coronary arteries (cardiac CT angiography).

CT is a sensitive method for diagnosis of abdominal diseases. It is used frequently to determine stage of cancer and to follow progress. It is also a useful test to investigate acute abdominal pain (especially of the lower quadrants, whereas ultrasound is the preferred first line investigation for right upper quadrant pain). Renal stones, appendicitis, pancreatitis, diverticulitis, abdominal aortic aneurysm, and bowel obstruction are conditions that are readily diagnosed and assessed with CT. CT is also the first line for detecting solid organ injury after trauma.