Genome editing is a process that involves several actions affecting DNA sequences in the genome, such as deletion, insertion or alteration of these sequences. Researchers in the field of genome editing spent many years attempting to create genome editing tools that were effective, easy-to-use and non-expensive.

These tools could be used for a wide array of applications, such as deleting the DNA sequences (gene) responsible for causing a particular genetic disorder, which would vastly improve gene therapy in humans. Another potential application would be for any industry that uses bacteria to create product yields. The genomes of these bacteria could be altered to optimize the amount of product they produce or the quality of that product. Finally, a third example of a potential application would be for agriculture. Using these tools to alter or manipulate a plant’s genome could be used to lessen or eliminate plant diseases, and they could be used to increase crop yields.

With all of these possibilities in the collective mind of the scientific community, it was a huge breakthrough when CRISPR was developed. CRISPR is a tool that operates on the molecular level and can edit DNA sequences to a very specific and accurate degree. It can be implemented to work on almost any locus. CRISPR has become extremely popular and is starting to be used as a standard genome editing tool in most research and development centres. CRISPR has unlocked a lot of potential for a variety of applications.
Before CRISPR

Nowadays, CRISPR is ubiquitous in the world of gene editing, but it was not the first method of altering a genome. The earliest methods came into use with the discovery of meganucleases and restriction enzymes.

The concept of accurate, specific genome editing was advanced by the development of zinc-finger nucleases (ZFNs) as a tool for gene editing. A ZFN is an enzyme with both a zinc finger DNA-binding domain and also a restriction endonuclease domain. The first domain is used to target and bind to specific sequences of DNA and the second domain is used to cleave the DNA at the target site. The zinc-finger domain is made of a 3-base pair segment of DNA that is designed to complement the target site and the restriction endonuclease cleaves the site that it is guided to by the first domain.

ZFN was a huge breakthrough for site-specific gene editing, but unfortunately it had some limitations. The results from ZFN use showed off-target effects, ZFNs are difficult to engineer, making them expensive for researchers and time-consuming for manufacturers. They were inefficient, and this meant that they could only be applied to achieve one target change at a time.

Years later, transcription activator-like effector nucleases (TALENs) were developed. This method of gene editing is similar to ZFNs, being composed of a DNA-binding domain and a second domain that cleaves the DNA. TALENs are more useful than ZFNs because their DNA-binding domains have more potential target sequences, meaning they can be used for more applications. TALENs were a step forward from ZFNs, but they were expensive to produce, despite because easier to design than ZFNs.

There is another gene editing technique that uses restriction enzymes along with recombinant adeno-associated viruses (rAAVs). The adeno-associated virus is a non-pathogenic virus infects mammalian cells and affects their genomes at sites that can be predicted. The virus genome can be altered to affect specific target DNA. The main limitation with rAAVs is that their vectors have a very limited amount of genetic material to be altered, and therefore their resulting effectiveness is limited. Their vectors are also difficult to produce.

These gene editing tools all had limitations. ZFNs and TALENs were challenging to design and manipulate. rAAVs were limited by their small vector capacities. CRISPR, on the other hand, is based on DNA-RNA interactions, which are commonly understood, and therefore, provides an easier, simpler way to edit genes. This simplicity and effectiveness has changed the field of gene editing forever.
CRISPR

CRISPR is made of two components which can be used to target the desired DNA sequence and then cleave the DNA at that site. It can be used by researchers to alter a specific gene. Examples of the uses of CRISPR include knocking out a gene, which means to make it inactive, or knocking in a gene, which could fix a deletion caused by mutation or add a beneficial trait to the genome.

CRISPR – History

It began in 1993, when researchers identified repetitive palindromic segments of DNA in prokaryotes. These segments were found among other sections of genetic material and were soon called Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). As it turned out, the sections of genetic material between the CRISPR sections were actually the interesting thing. This discovery built the foundation for the creation of CRISPR-Cas9 technology.

In 2007, a leap forward was made when researchers realised that the function of CRISPR was for prokaryotic immunity. The underlying process, at a molecular level, was not fully understood for another 5 years. Both bacteria and archaea (the prokaryotes) use CRISPR-Cas9 to fight off invading viruses. When the viral infection occurs, the prokaryotic cell uses Cas9 (which is a CRISPR-associated nuclease) to cleave the viral DNA by creating a double-strand break (DSB) in the target DNA sequence.

The Cas9 molecule is guided to the target DNA by a guide RNA (gRNA). This short RNA fragment (the gRNA) is complementary to the target viral DNA sequence. This specific guiding system allows the Cas9 to cleave the DNA at a very specific level. This cleaving process destroys the virus. Additionally, a piece of the foreign DNA (referred to as a “spacer”) can be retained by the prokaryote and stored. The spacer will be kept in between the palindromic sequences of the CRISPR segments, which allows the prokaryote to retain a memory of previous infections. In this way, any attempted re-infection by the virus would be rapidly prevented and the attacking virus would be destroyed. This is basically the equivalent of the human immune system, which takes and retains antigens to prevent re-infection.

Researchers quickly understood, after figuring out the CRISPR mechanism in prokaryotes, that it could have massively beneficial uses in humans, other animals, plants and microbes.
CRISPR Components

CRISPR has two components – a guide RNA (gRNA) and a CRISPR-associated endonuclease (Cas). The gRNA is specific to the target DNA sequence. In experiments using CRISPR, the gRNA and Cas are combined and the result is a ribonucleoprotein (RNP) complex.

Cas is a protein that acts to cut the target DNA while the gRNA guides it to the target DNA site. In prokaryotes, the gRNA is used to target viral DNA, but as a gene editing tool, it can be designed to target any gene site in almost any location.

Cas9 is a popular choice of Cas proteins. Cas9 is from Streptococcus pyogenes. The gRNA finds the target site and binds to the DNA, but this binding requires the presence of a PAM (protospacer adjacent motif) immediately downstream of the target but on the opposite DNA strand. Different Cas proteins (from different prokaryotic species) recognise different PAM sequences. Cas9 is popular because of the frequency and flexibility of its PAM, which is 5’-NGG-3’. The N represents any nucleotide. This means that any DNA sequence with two G (guanine) bases can be used to form a PAM for Cas9.

If the gRNA successfully binds to the target, the Cas9 cleaves both DNA strands. This cleaving process takes place 3 to 4 nucleotides upstream of the PAM site.

Figure 1.

CRISPR-Cas9 is composed of a gRNA and Cas9 nuclease. Together, these create a ribonucleoprotein (RNP) complex. A specific PAM must be present in the genomic DNA for the gRNA to be able to bind to the target DNA segment. The Cas9 makes a double-strand break (DSB). Endogenous repair mechanisms react to the DSB and a frameshift mutation may cause gene knockout, or they could cause knock-in of a designated sequence. For knock-in, a DNA template must be present.
In nature, gRNA is made of two separate sections of RNA. These two sections are CRISPR RNA (crRNA) and transactivating CRISPR RNA (tracrRNA). crRNA is 18-20 base pairs in length and binds to the DNA target sequence. TracrRNA acts as a structure which the crRNA-Cas9 interaction can take place on. In nature, the gRNA is a duplex molecule, with crRNA and tracrRNA annealed together. Synthetically, gRNA can be produced with these two molecules connected by a linker loop. These are called single guide RNAs (sgRNAs).

Cas9 carries out its role in CRISPR-Cas gene editing experiments by causing a DSB to form in the DNA (Fig. 3). This resembles the process in prokaryotes. The DSB formation is the first step in the CRISPR editing process. It is followed by a repair mechanism which decides what type of gene editing will occur. Two types of repair will be discussed next, namely non-homologous end joining (NHEJ) and homology-directed repair (HDR).
As previously mentioned, CRISPR causes DSBs at desired sites in the genome. This is just the first step in the editing process. It is the repair mechanisms that follow this that allow the editing to occur. Innate DNA repair processes automatically respond to the formation of the DSB. The two main types of repair mechanisms that are used to edit genes are non-homologous end joining (NHEJ) and homology-directed repair (HDR).

Non-Homologous End Joining (NHEJ)

NHEJ can be used when the desired result is to permanently knockout a gene (so that no functional protein is made). (Fig. 4). NHEJ facilitates the re-joining of the DNA ends. However, it often allows erroneous changes to occur, which may result in inserted or deleted nucleotides that are not intended. These are called indels. If the number of nucleotide changes (inserted or deleted) are not a multiple of three, a frameshift mutation will occur. This mutation will likely eliminate the functionality of the resulting protein.

Homology Directed Repair (HDR)

If the gene editing result desired by the researchers is to replace the targeted DNA sequence with another sequence, then HDR can be used. A DNA template from a donor that possessed that desired DNA segment is introduced. This template is surrounded by sections of homologous DNA sequences. The host’s repair mechanisms will use this template to fix the DSB by using homologous recombination. By this process, the donor’s sequence is incorporated into the sequence being repaired.
Components of CRISPR-Cas9

Understanding CRISPR – The Basics

Figure 5.

- NHEJ REPAIR
  - Insertion
  - Deletion
  - Homology-Directed Repair

- Cas9-Mediated Double-Strand Break (DSB)

- DNA Donor Template
  - Knock-In Sequence
What is CRISPR Capable of?

The field of gene editing has grown vastly since CRISPR was developed. CRISPR can be used in a very large variety of cells in many different organisms, such as mammals, plants and fungi. It has been used to alter genomes in many ways, including changes in nucleotide sequences and changing the expression of genes. Figure 6 shows some of the current uses of CRISPR.

CRISPR is being used for many applications in gene editing, not just knocking in and knocking out genes. Research into CRISPR and development of new CRISPR methods has led to several new applications of the technique. Examples include CRISPRi and CRISPRa, anti-CRISPR proteins, CRISPR screens, and tagging genes via CRISPR to allow for tracking and visualization.
A knockout refers to the process of making a gene permanently inactive, which means that it cannot encode a functioning protein. CRISPR can cause this because of how error-prone NHEJ can be. As previously described, NHEJ often produces indels that can result in a frameshift mutation and inactivate the function of the gene. A particularly susceptible DNA sequence is a premature stop codon.

Knockouts are also results of using multi-guide sgRNAs. These are gRNAs that target the same gene. The subsequent multiple cuts in the DNA induces at least one large fragment deletion in the target DNA. These deletions cause the loss of several amino acids, which will likely result in the complete inactivation of the target gene.

The resulting changes in protein expression following a knockout can provide researchers with an insight into the phenotype of a cell or organism. This is useful for many applications, such as identifying and validating potential drug targets, analysing cellular mechanisms and evaluating antibody activity.

Knockings is the process of incorporating genetic material into a host genome. This process is facilitated by HDR, as discussed previously. In experiments using HDR, a DNA template is introduced to allow the HDR mechanism to introduce the desired change into the host genome. HDR, as a gene editing tool, allows for many applications. Examples range from single point mutation alterations to the addition of selectable markers. The HDR mechanism still requires further research to improve efficiency, but researchers have managed to use it to fix a mutation which causes cataracts in mice. This illustrates that HDR is a viable concept for correcting genetic diseases.
CRISPR interference (CRISPRi)

Knocking out is one way to disrupt gene expression by a particular DNA sequence, but gene expression can also be suppressed in another way that does not require the corresponding DNA to be altered. In 2013, Qi et al. developed a variant of Cas9 that does not cleave DNA. This was achieved by altering the endonuclease domains through mutation. This new variant is called dead Cas9 or dCas9. Using dCas9, a new technique was created where the dCas9 binds to the target DNA (but does not cleave it) and prevents the host cell’s transcription machinery from reaching the promoter. This prevents the gene from being expressed without cleaving the DNA. (Fig. 7a.) Combining a transcriptional repressor domain with dCas9 produces a mechanism that is reversible and effective in inhibiting gene expression.

The term CRISPR interference is a reference to its precursor, the gene-silencing technique RNA interference (RNAi). While RNAi destroyed RNA transcripts to silence gene editing, CRISPRi affects genes by impacting DNA. CRISPRi has higher efficiency, versatility and less unintended (off-target) effects than RNAi.

CRISPR activation (CRISPRa)

dCas9 endonucleases can silence gene expression but they can also be used to activate desired genes (called CRISPR activation). Combining a transcriptional activator with dCas9 can allow for editing that results in target gene overexpression.
CRISPR can be used for functional screening of the genome. RNAi was the main method used for screening to test for gene loss of function. This screening process involved the systematic inhibition of genes across the genome to ascertain their function. RNAi has many problems, such as low efficiency and a high number of off-target effects. CRISPR is far more effective, with gRNA libraries that are being developed to allow knocking out of hundreds of genes with high efficiency in one screening.

Anti-CRISPR

CRISPR-Cas9 allows the fine-tuning of genomic DNA. However, there is a downside. There is a risk of off-target effects, such as cleaving the DNA in the wrong location. The solution is to use anti-CRISPR proteins that inhibit the activity of Cas9. This can be seen in nature, when bacteriophages use these types of proteins to deflect the prokaryote’s CRISPR machinery. This technology can be applied to decrease errors in the editing process. When the anti-CRISPR machinery is introduced after the editing process occurs, the cleavage at on-target sites is only partially decreased, while cleavage at off-target sites is greatly decreased.

Figure 8. Systematically disrupt a set of genes using a CRISPR library

BioRelay
CRISPR can be used to visualize regions of the genome. This can be attained by the attachment of fluorescent proteins, such as green fluorescent protein (GFP), to dCas9 or gRNAs, and then tagging the target areas of the genome with CRISPR. This allows researchers to visualize nucleic acids, a process which was previously difficult to achieve in real time.

### Steps

**Design**

An experiment with CRISPR begins with organising the parameters and components for it. The gRNA must be designed and the suitable Cas nuclease must be selected. Once they are completed, it is necessary to select the CRISPR component format, choose a method of transfection, and prepare the optimal conditions for the cell type being used. These steps will give a strong likelihood for the success of the gene editing experiment.

**Edit**

After designing the experiment, you can begin to introduce the target-specific gRNA and Cas9 nuclease to the cells. Carry out the transfection and allow CRISPR to act.

**Analyze**

When the editing is completed, analyse the relevant genomic sequences to find out the frequency and type of edits that have occurred in the genome. Several analysis tools exist that can be used to evaluate the genomic results and ascertain the editing efficiency. Protein and phenotypic assays are available to evaluate gene editing effectiveness.
CRISPR is extremely popular because of how specific and feasible it is as a tool for genome editing. CRISPR has vastly advanced and redefined the field of genome engineering. CRISPR has facilitated many advances in recent years. Researchers are attempting to develop the technology to reveal its huge potential.

There is a modified version of CRISPR that is being used to investigate epigenomics. This is a family of chemical groups that are found throughout DNA and its associated histone proteins. Previous to this CRISPR advancement, researchers were limited to studying the correlation between gene expression and epigenetic markers. This CRISPR complex can acetylate histone proteins at desired locations. This can be used to reveal more information about the causal relationship between gene expression and epigenetic markers.

CRISPR is becoming more important in the biomedical industry. Drug discovery and development is renowned as a long, expensive and difficult process, but with CRISPR it is believed that the pre-clinical stage will become easier. An example is CRISPR screening libraries which are now available to find new drug targets. Also, CRISPR can help to create accurate disease models for drug development, as well as CRISPR research for in vivo and ex vivo therapies. Gene editing could greatly benefit the development of improved therapies and medicines in the future.

CRISPR is advancing research into the most problematic areas of the field of biomedical science. It is also facilitating advances in other fields of science, such as human therapeutics, agriculture, biofuels and general scientific research.