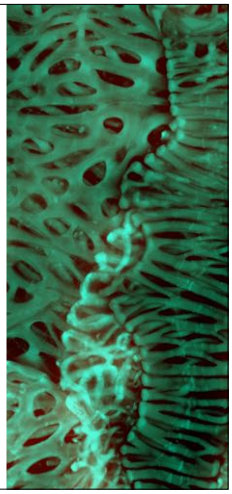
Genomics, Epigenetics & Synthetic Biology

Lecture 2:
Synthetic Biology and DNA engineering



Jim Haseloff
www.haseloff-lab.org (Education)

1

What is it?
Creation of artificial life?
Extension of genetic engineering?

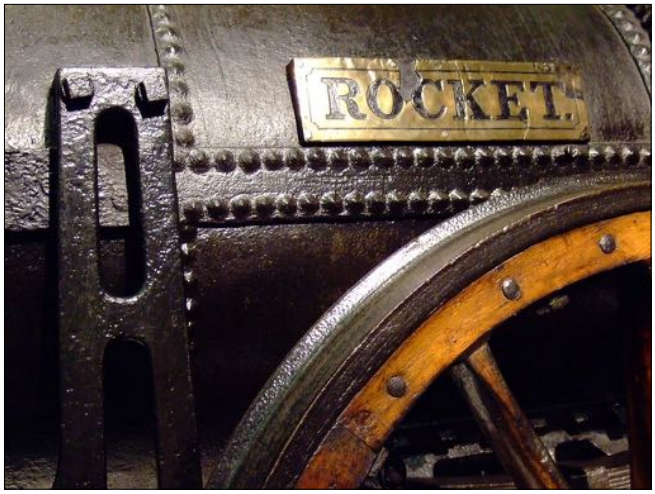
syn·thet·ic [sin-**thet**-ik] –adjective

1. Prepared or made artificially, not of natural origin.

2. Relating to, or involving synthesis (construction of a coherent whole from separate elements)

2

What is synthetic biology? The word "synthetic" can mean (i) artificial or (ii) relate to synthesis or construction. We will look at how the latter meaning can be used to refer to systematic approaches to biological construction. But, first we will look at the emergence of engineering in different fields.



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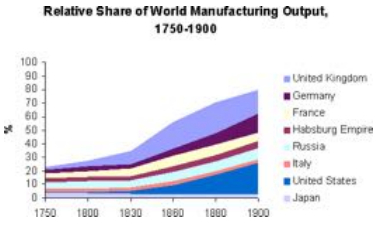
The Industrial Revolution was based on innovations in coal, iron, steam and mechanical engineering that took place in the mid-to-late 1700s. This led to inventions in the early 1800s, like the first modern steam engine found in Stephenson's Rocket.

First phase of the Industrial Revolution: innovation

- **Steam power** - Improved steam engines were initially used for pumping out mines, but from the 1780s were applied to power machines. This enabled rapid development of efficient semi-automated factories
- **Iron founding** - Coke replaced charcoal in iron smelting. Improved production of bar iron, and eventually steel, resulted.
- **Textiles** - Cotton spinning was revolutionised by the invention of Richard Arkwright's water frame, James Hargreaves's Spinning Jenny, and Samuel Crompton's Spinning Mule). Similar technology was applied to spinning worsted yarn for various textiles and flax for linen.

Second phase of the Industrial Revolution: manufacturing



(Paul Bairoch, "International Industrialization Levels from 1750 to 1980")

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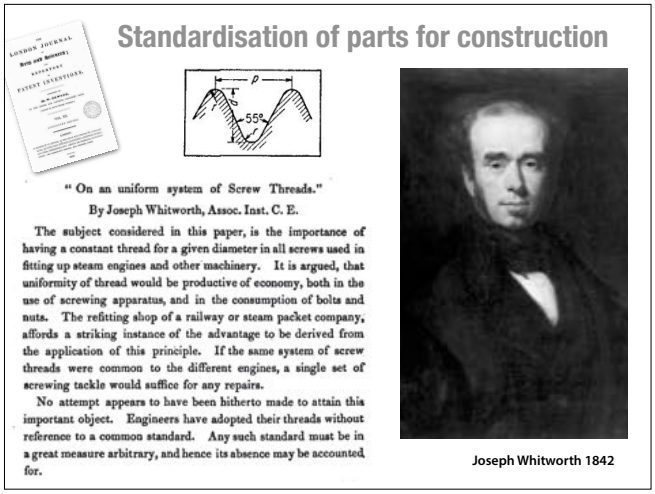
In mechanical engineering there was a lag phase between the periods of innovation and emergence of applications in manufacturing industries. The Industrial Revolution first took root in the United Kingdom. However major impacts on industrial output were first seen towards the mid-1800s.



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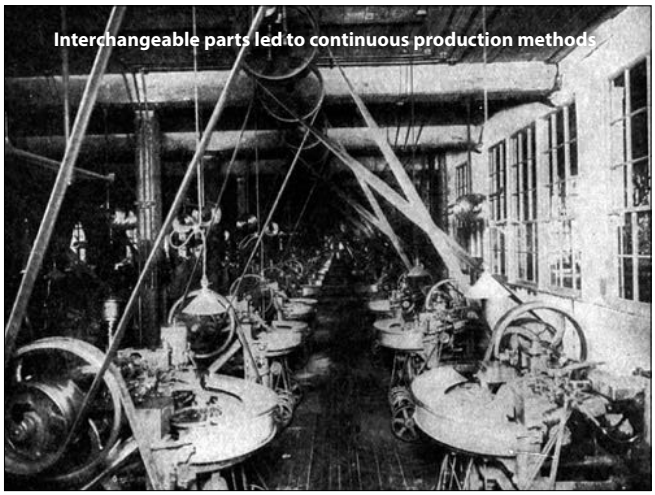
The application of new mechanical engineering principles in industry was accompanied by standardisation. For example, Whitworth proposed the first widely accepted standard screw threads for mechanical fasteners in 1830. Before this time, mechanical engineers needed to machine their own bespoke fasteners.



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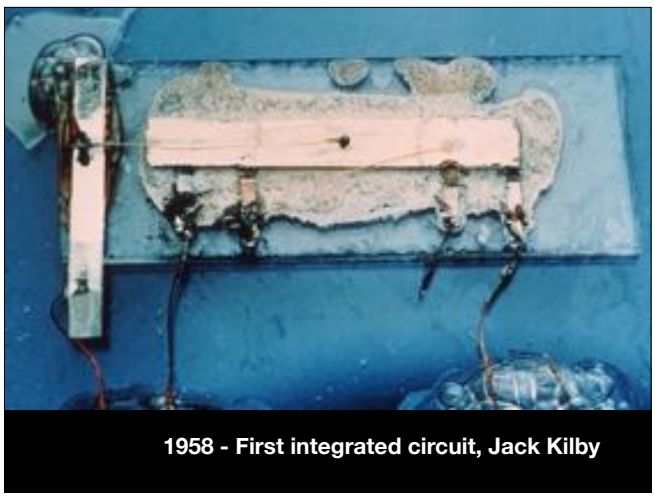
The adoption of standards in mechanical engineering allow the use of interchangeable parts and facilitated the development of continuous production methods, and increased industrial output.



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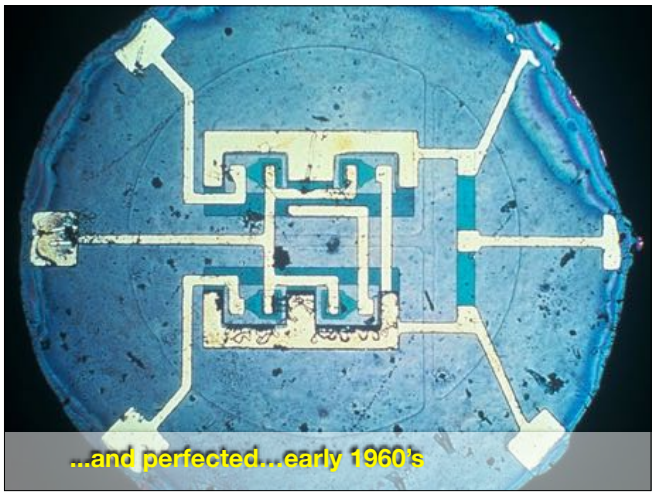
The first microelectronic devices were crude and handmade.



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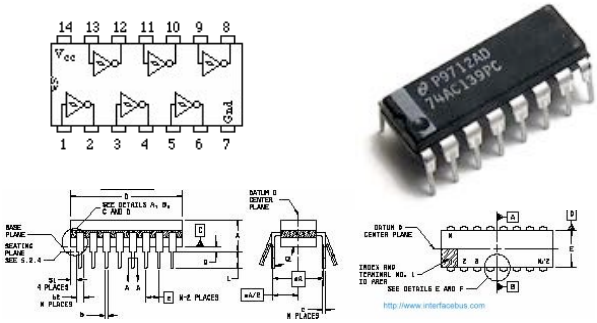
Within a few years the combination of new the graphic and planar transistor techniques had created recognisable prototypes of the devices that we recognise today.

1. Standardisation of parts



Standard mechanical and electrical interfaces were established for integrated devices by the early1960's, and form the basis for today's microelectronics industry

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Within a few years mechanical and electrical interfaces had been standardised. This allowed the interoperability of these devices, and for engineers to mix devices from different sources.

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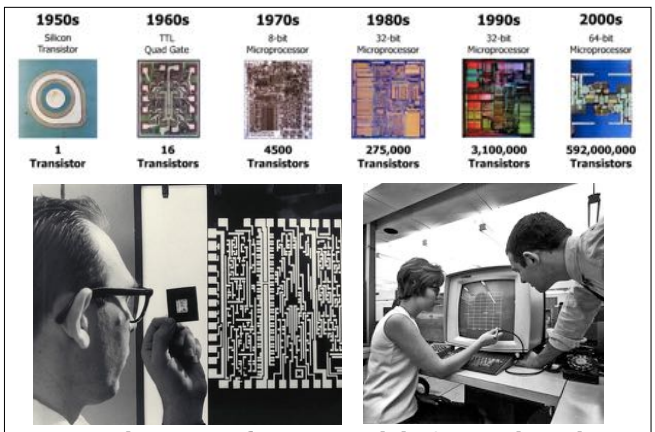
At first, these devices were designed by hand.



2. Development of automated design tools and modular circuits to deal with increased complexity

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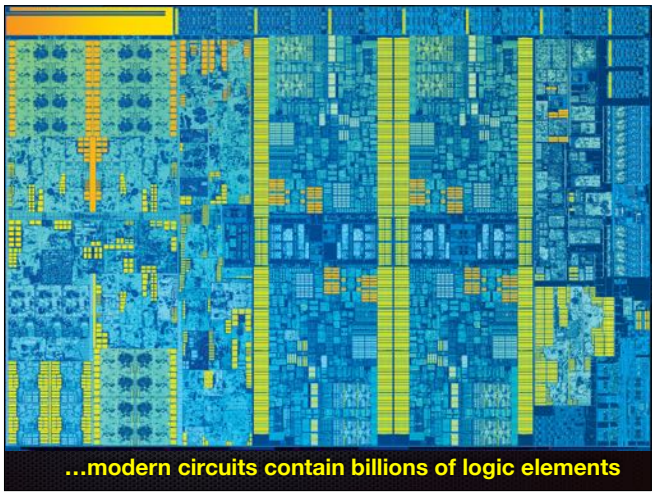
Increasing complexity saw the emergence of new automated design tools and reusable modular elements.



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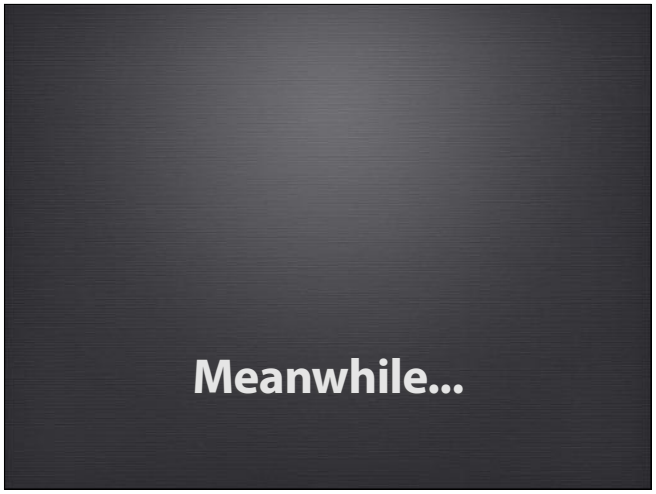
Modularisation and standardisation are the hallmarks of modern engineering. They allow management of highly complex systems.



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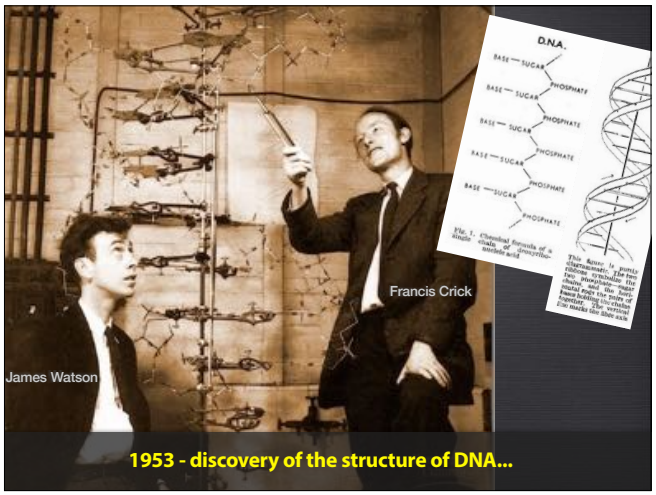

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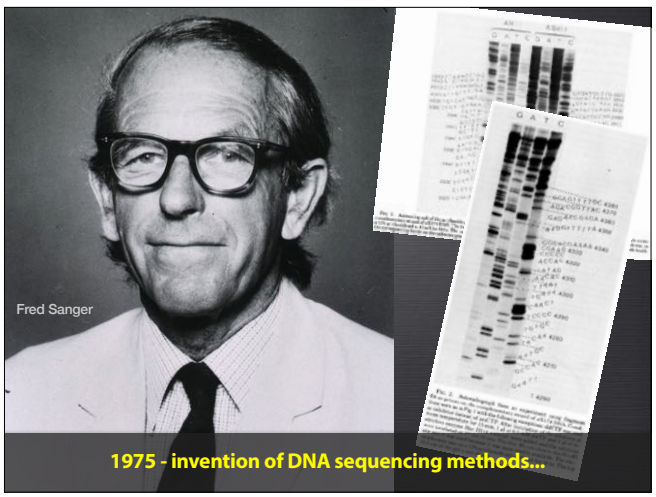
Over roughly the same time period, we have seen basic innovations in biology that allow similar engineering approaches. From discovery of the structure of DNA in 1953…



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… To the development of DNA sequencing methods - at the kilobase-scale with Sanger sequencing in 1975

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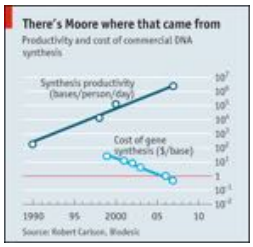
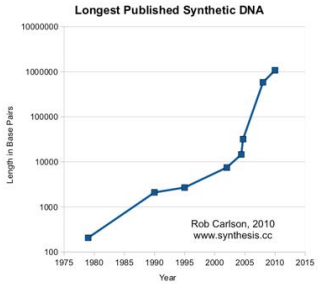
… Through to today's next generation gigabase-scale sequencing efforts.

Enabling DNA Technologies

DNA Sequencing, DNA Assembly, DNA Synthesis &
Computational Modelling & Genetic Design





'Synthetic Biology - Life 2.0'
The Economist, August 31st 2006

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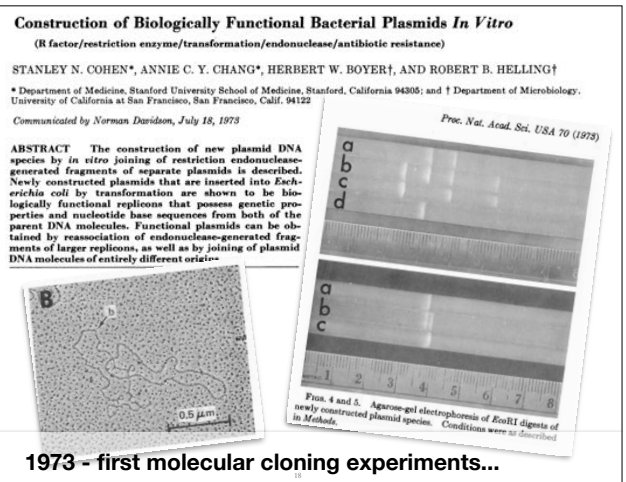
Not only has the speed of DNA reading improved at an exponential rate, but the technology of DNA synthesis has also improved. It is now possible to synthesise DNA at dramatically lower prices, pennies per base-pair.



Construction of Biologically Functional Bacterial Plasmids *In Vitro*

(R factor/restriction enzyme/transformation/endonuclease/antibiotic resistance)

STANLEY N. COHEN*, ANNIE C. Y. CHANG*, HERBERT W. BOYER†, AND ROBERT B. HELLING†

* Department of Medicine, Stanford University School of Medicine, Stanford, California 94305; and † Department of Microbiology, University of California at San Francisco, San Francisco, Calif. 94122

Communicated by Norman Davidson, July 18, 1973

ABSTRACT The construction of new plasmid DNA species by *in vitro* joining of restriction endonuclease-generated fragments of separate plasmids is described. Newly constructed plasmids that are inserted into *Escherichia coli* by transformation are shown to be biologically functional replicons that possess genetic properties and nucleotide base sequences from both of the parent DNA molecules. Functional plasmids can be obtained by reassociation of endonuclease-generated fragments of larger replicons, as well as by joining of plasmid DNA molecules of entirely different origin...

Proc. Nat. Acad. Sci. USA 70 (1973)

FIGS. 4 and 5. Agarose-gel electrophoresis of EcoRI digests of newly constructed plasmid species. Conditions were as described in *Methods*.

1973 - first molecular cloning experiments...

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The first molecular cloning experiments were published in 1973. In these first experiments DNAs were cut with restriction endonucleases, separated by electrophoresis, and pasted together with T4 DNA ligase. These experiments have triggered decades of genetic engineering experiments.

But...bespoke DNA assembly techniques are still common practice in the field after 40 years

Smithsonian museum exhibit: Stanley Cohen's laboratory bench

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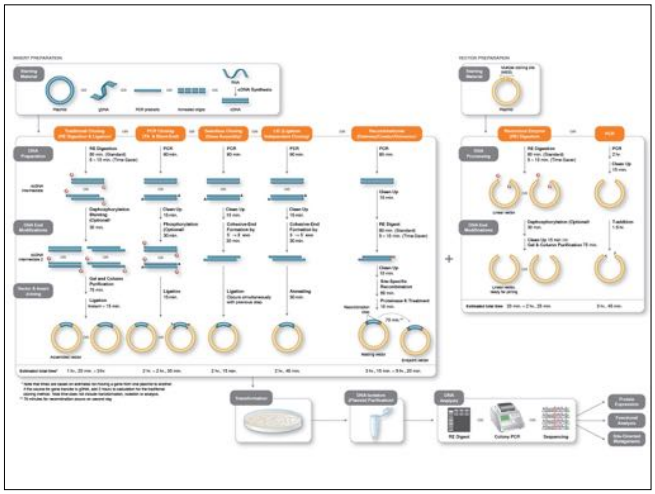
Photograph of the reconstruction Stanley Cohen's laboratory bench in the Smithsonian Museum. Not dissimilar to a modern molecular biologist's bench.



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Over subsequent decades a large variety of DNA cloning techniques have been invented. In all these, cases the cloning strategy is bespoke, adapted to the target sequence either by choice of reagents like restriction enzymes, or by the design of DNA adapters of different types.

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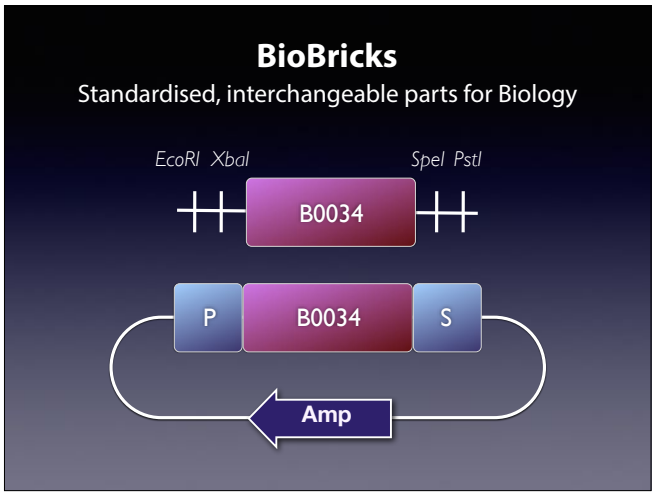
Tom Knight, a computer scientist at MIT, proposed a generalised method for large-scale DNA assemblies:

Idempotent Vector Design for Standard Assembly of Biobricks (2003)

The lack of standardization in assembly techniques for DNA sequences forces each DNA assembly reaction to be both an experimental tool for addressing the current research topic, and an experiment in and of itself. One of our goals is to replace this ad hoc experimental design with a set of standard and reliable engineering mechanisms to remove much of the tedium and surprise during assembly of genetic components into larger systems. http://hdl.handle.net/1721.1/21168



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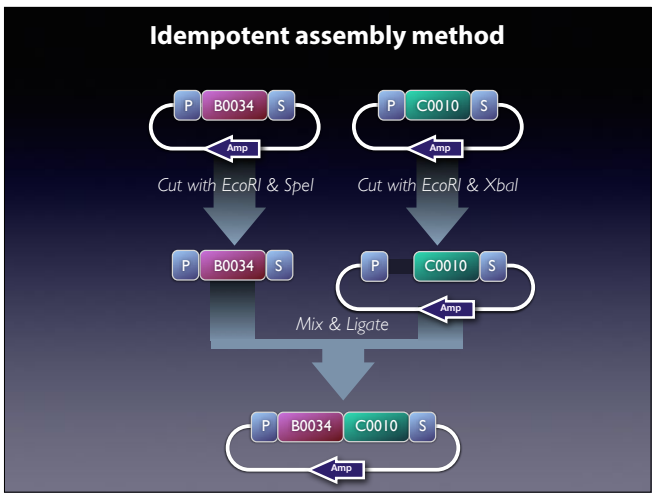
DNA parts would be composed in a standardised format for modular assembly. The modular parts would therefore be interchangeable, and…



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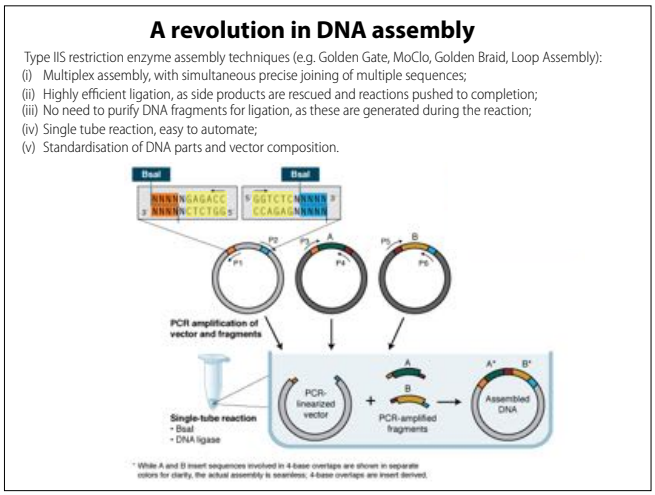
…the combination of any two parts would recreate the format of a standard part. (An object's properties remains unchanged during an idempotent operation). Note the arrangement of prefix (P) ands suffix (S) elements in this diagram, as two fragments are ligated.



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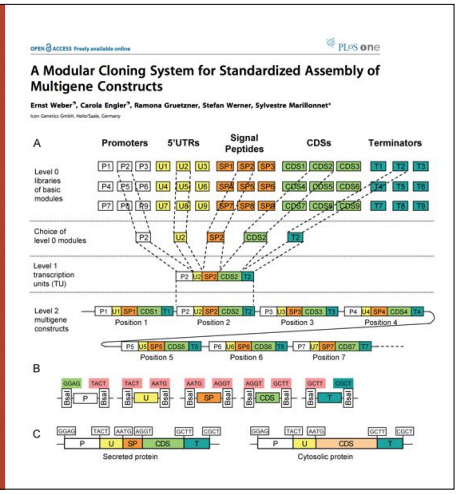
This approach has become increasingly sophisticated, now in the form of type IIS assembly techniques. These rely on restriction enzymes with cleavage sites that are offset from their recognition sequence. There is no need to isolate DNA fragments. Intact plasmid DNAs can be mixed, and cleavage and ligation of the fragments occurs in a single tube reaction to create the expected product.

Type IIS DNA assembly protocols:

Golden Gate
MoClo
ENSA
Golden Braid:

adopted by the plant research community



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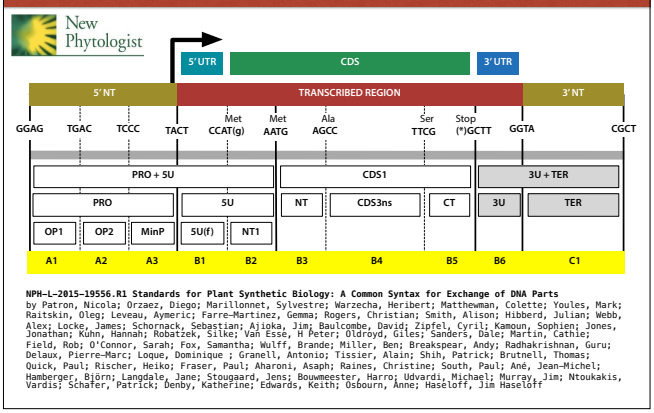
Type IIS assembly relies on the formatting of DNA fragments into particular classes. The different class fragments are then ligated to produce transcription units and can be further combined into a large multi-gene assemblies. The efficiency and ease of the assembly reactions has meant that this technique has been widely adopted by the plant research community.

A common syntax for plant DNA parts

Based on Golden Gate standard assembly and type IIs restriction enzyme splints.



NPH-L-2015-19556.R1 Standards for Plant Synthetic Biology: A Common Syntax for Exchange of DNA Parts
by Patron, Nicola; Orzaez, Diego; Marillonnet, Sylvestre; Warzecha, Heribert; Matthewman, Colette; Youles, Mark; Raitskin, Oleg; Leveau, Aymeric; Farre-Martinez, Gemma; Rogers, Christian; Smith, Alison; Hibberd, Julian; Webb, Alex; Locke, James; Schornack, Sebastian; Ajioka, Jim; Baulcombe, David; Zipfel, Cyril; Kamoun, Sophien; Jones, Jonathan; Kuhn, Hannah; Robatzek, Silke; Van Esse, H Peter; Oldroyd, Giles; Sanders, Dale; Martin, Cathie; Field, Rob; O'Connor, Sarah; Fox, Samantha; Wulff, Brande; Miller, Ben; Breakspear, Andy; Radhakrishnan, Guru; Delaux, Pierre-Marc; Loque, Dominique ; Granell, Antonio; Tissier, Alain; Shih, Patrick; Brutnell, Thomas; Quick, Paul; Rischer, Heiko; Fraser, Paul; Aharoni, Asaph; Raines, Christine; South, Paul; Ané, Jean-Michel; Hamberger, Björn; Langdale, Jane; Stougaard, Jens; Bouwmeester, Harro; Udvardi, Michael; Murray, Jim; Ntoukakis, Vardis; Schäfer, Patrick; Denby, Katherine; Edwards, Keith; Osbourn, Anne; Haseloff, Jim Haseloff

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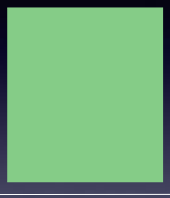
Further, plant researchers have adopted a common syntax for these plant parts to ensure interoperability across the community.

Abstraction

Insulate relevant characteristics from process from excessive details



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These advances have facilitated the efficient construction of engineered DNA sequences in a technical way. They also allow researchers to regard DNA encoded functions in a modular fashion. For example, this DNA part encodes the sequence of the green fluorescent protein. The modular nature of assembly standards can help insulate the designer from the underlying molecular-scale details of the DNA part.

Standardisation

Construction from "off the shelf" parts with known characteristics

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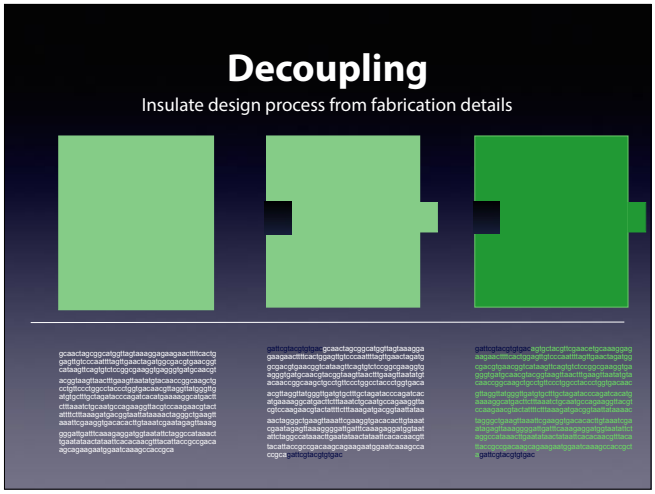
Standardised parts come with an implied means of assembly. They can "plug" together in a manner similar to Lego parts.



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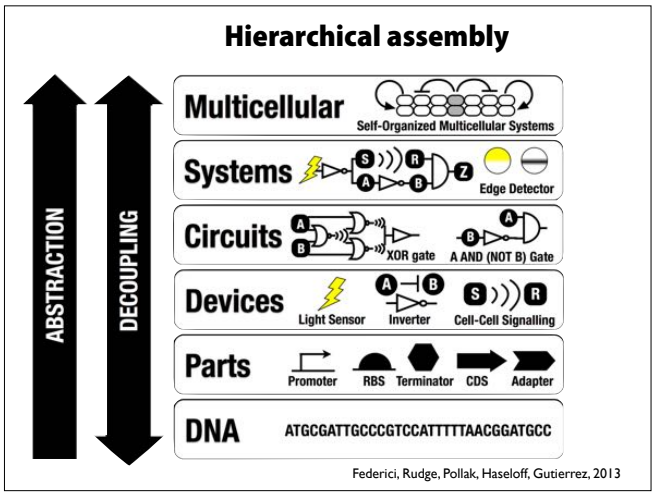
The process of improvement of DNA parts can to be separated from the design process. In this case, a new part with a modified coding sequence for a brighter green fluorescent protein can be used interchangeably by a genetic circuit designer. The design process is decoupled from the fabrication process.



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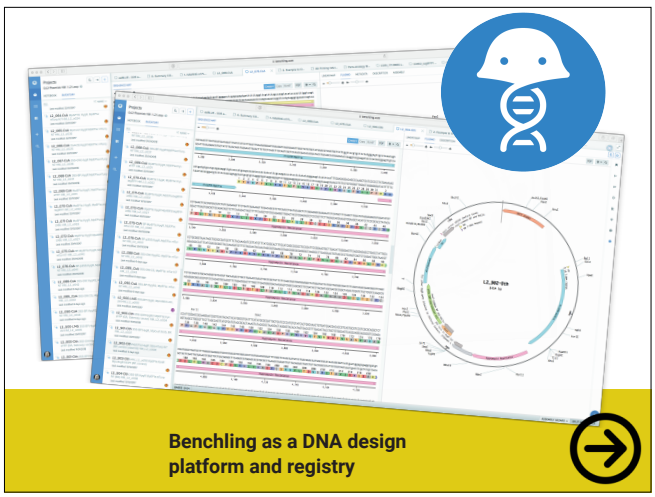
The introduction of these engineering principles in biology is leading towards a more hierarchical way of constructing complex systems. DNA encoded functions can be formulated as standardised parts. These parts can be assembled into devices circuits and genetic systems - which can in turn be installed in multicellular systems.



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As is the case in other engineering fields, we are seeing the emergence of software tools for information exchange automated design and analysis.

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Standardisation of DNA parts has fostered the emergence of new agencies for global sharing and distribution.

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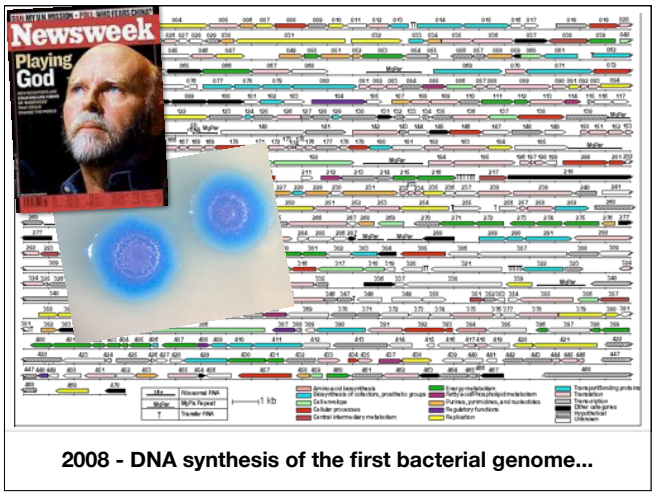
We also seeing the emergence of legal frameworks to facilitate sharing of standardised DNA parts.



2008 - DNA synthesis of the first bacterial genome...

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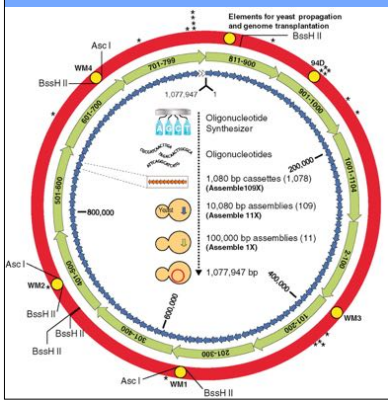
In addition to the assembly of genetic systems from standardised parts, we are also seeing the re-factoring, synthesis and transplantation of entire genomes.

Chemical synthesis of a Bacterial Genome: *Mycoplasma mycoides*, JCVI 2010



Assembly of *M. mycoides* genome

1. Overlapping oligonucleotides (including yeast vector, *lacZ*) recombined to make 1080 bp cassettes (orange arrows).

2. In sets of 10, the cassettes recombined to produce 109 ~10kb assemblies (blue arrows)

3. In sets of 10, the ~10kb assemblies recombined to produce 11 ~100kb assemblies (green arrows)

4. These 11 assemblies were recombined to the final genome, sMmYCp235 synthetic genome to create JCVI-syn1.0 cell line

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The entire *Mycoplasma mycoides* genome was synthesised in the form of small oligonucleotides. These were stitched together to form one kilobase-sized cassettes, which were in turn assembled into first 10kb, and then 100 kb fragments. These were finally assembled into the megabase circular genome of the bacterium. Small fragments were assembled *in vitro*, the larger fragments were assembled by homologous recombination *in vivo*, using yeast as a host.

Gibson assembly: a breakthrough in large-scale, rapid DNA assembly

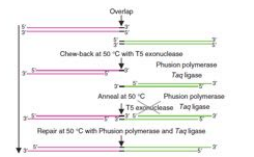


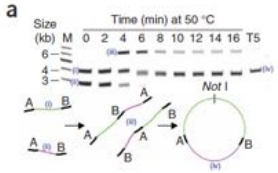
Figure 1 | One-step isothermal *in vitro* recombination. Two adjacent DNA fragments (magenta and green) sharing terminal sequence overlaps (black) were joined into a covalently sealed molecule in a one-step isothermal reaction. T5 exonuclease removed nucleotides from the 5′ ends of double-stranded DNA molecules, complementary single-stranded DNA overhangs annealed, Phusion DNA polymerase filled the gaps and *Taq* DNA ligase sealed the nicks. T5 exonuclease is heat-labile and is inactivated during the 50 °C incubation.

Enzymatic assembly of DNA molecules up to several hundred kilobases

Daniel G Gibson[1], Lei Young[1], Ray-Yuan Chuang[1], J Craig Venter[1,2], Clyde A Hutchison III[2] & Hamilton O Smith[2]

We describe an isothermal, single-reaction method for assembling multiple overlapping DNA molecules by the concerted action of a 5′ exonuclease, a DNA polymerase and a DNA ligase. First we recessed DNA fragments, yielding single-stranded DNA overhangs that specifically annealed, and then covalently joined them. This assembly method can be used to seamlessly construct synthetic and natural genes, genetic pathways and entire genomes, and could be a useful molecular engineering tool.

Nature Biotechnology, May 2009



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The task of assembling a complete bacterial genome was made possible by a number of technical innovations. First, an efficient technique for the multiplex assembly of scar free DNA fragments was developed. This was called Gibson assembly, after the inventor.

Large DNA fragment assembly via homologous recombination in yeast

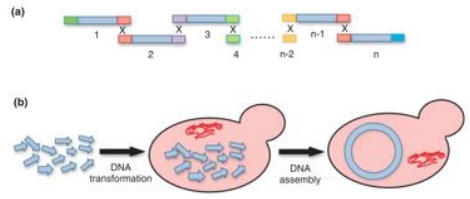

FIGURE 2 | Synthesis of large DNA molecules in yeast. (a) Yeast homologous recombination mechanism. DNA fragments sharing an overlap region at 3'- and 5'-ends with the neighboring DNA fragments can be assembled into a single larger DNA molecule. (b) Construction of a synthetic *M. genitalium* genome. Twenty-five different overlapping DNA segments (blue arrows, 17–35 kb each) composing the genome were co-transformed into yeast followed by assembly of the entire genome in a single step.

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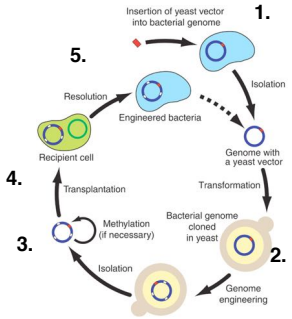
Second, large DNA fragments could be assembled in an ordered fashion using homologous recombination in yeast. This technique is capable of producing chromosome scale synthetic DNAs.

Creating Bacterial Strains from Genomes that have been cloned and engineered in yeast



Transplantation of *M. mycoides* genome via yeast engineering

1. Insertion of yeast vector into bacterial genome
2. Transform yeast with entire genome, engineer in yeast
3. Isolate DNA, protect (methylation)
4. Transform recipient bacteria
5. Resolve (loss of recipient genome)

gue et al. (2009) Science 325: 1693-1696

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Third, the judicious inclusion of sequences that allowed replication and maintenance of foreign DNAs in yeast - allowed the propagation, manipulation isolation of chromosome-scale synthetic DNAs. The synthetic bacterial chromosomes could then be isolated from these yeast strains.

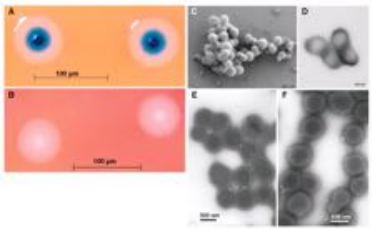
Transplantation of a synthetic bacterial genome: *Mycoplasma mycoides*, JCVI 2010

Genome transplantation

DNA from the final assembly in yeast sMmYCp235 synthetic genome was transplanted into a *M. capricolum* cell to ultimately produce JCVI-syn1.0

A&B. WT *M. mycoides* colonies are white, JCVI-syn1.0 are blue (*lacZ*/beta galactosidase + Xgal). "Fried egg" morphologies characteristic of mycoplasma species.

C,D,E & F. Electron micrographs of cells. Both WT and JCVI-syn1.0 show the same morphology



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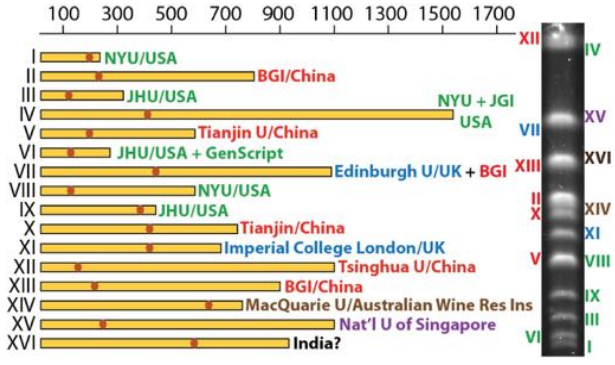
Synthetic bacterial chromosomes could then be transplanted into cells of related bacterial species after the destruction of the endogenous genome. Thus *Mycoplasma capricolum* could be converted to a synthetic version of *Mycoplasma mycoides*.

SC2.0 Synthetic Yeast Genome Project



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Work is now underway on a much larger project: to create synthetic versions of the yeast (*Saccharomyces cerevisiae*) genome. This is an international project where individual chromosomes have been parcelled out to different institutions.

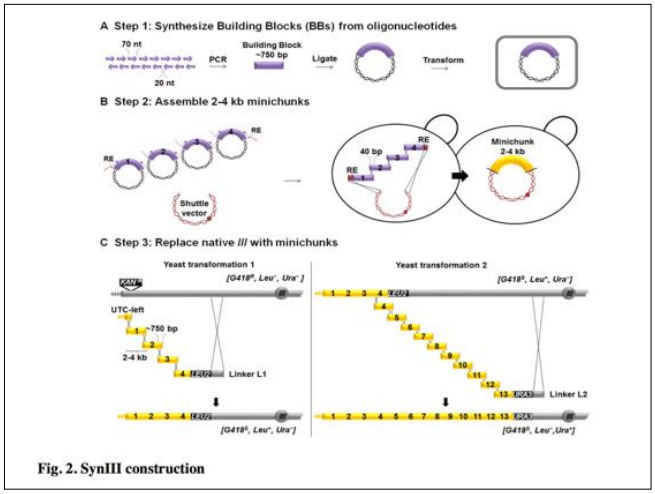

Fig. 2. SynIII construction

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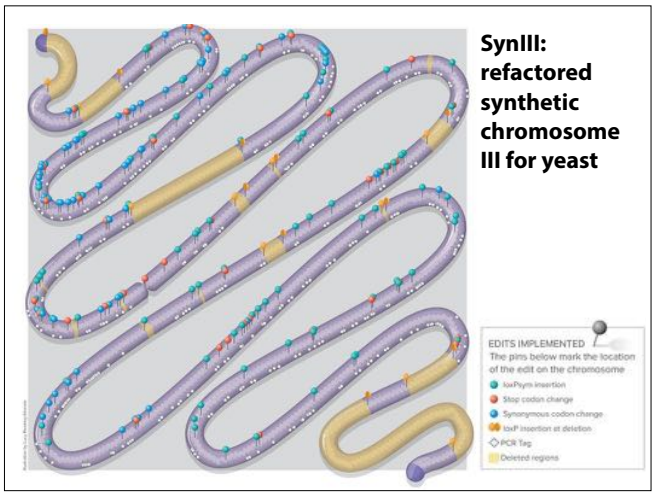
In a similar approach to the artificial bacterial chromosomes, small synthetic oligonucleotides are successively pieced together to create larger DNA fragments which in turn are progressively assembled into larger fragments or directly recombined into the target chromosome. Using alternative selection markers linear stretches of use chromosomes are progressively converted to the synthetic version. The refactoring of the 12 megabase yeast genome is largely complete. (http://www.syntheticyeastresource.com)



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Schematic diagram of the refactored yeast chromosome III - showing (i) the introduction of Lox sites for scrambling the genome, (ii) altered codon usage, (iii) introduction of specific PCR tags and (iv) deletion of non-essential regions.

Multi-scale DNA assembly methods

Table 2. Technical specifications of several DNA assembly methods.

Assembly methods	Fragment overhangs	Typical number of fragments for assembly	Demonstrated size of assembled construct (kb)	Efficiency	References
Gibson	40 bp overlaps	c. 4	900	90% (for 3 fragments)	Gibson et al. (2009)
In-fusion	>15 bp overlaps	2–3	c. 5	>60% (for 2 fragments) <40% (for 3 fragments)	Sleight et al. (2010)
USER	7–12 bp overlaps, must contain one dU at the base	3–7	c. 8	>90% (for up to 7 fragments)	Jensen et al. (2014) Lund et al. (2014)
CPEC	15–25 bp overlaps	>4	c. 8	95–100%	Quan and Tian (2009)
MoClo	4 bp overlaps and recognition site for type IIS restriction enzyme	c. 10	33 (in three rounds)	>90%	Engler et al. (2009) Weber et al. (2011)
LCR	Fragments must be 5′-phosphorylated, 60- to 90-bp-long bridging oligos are also needed	>10	20	>90% (up to 6 fragments) c. 75% (12 fragments)	de Kok et al. (2014)
In vivo recombination in S. cerevisiae	>40 bp overlaps	>10	>20	>90% (up to 6 fragments) c. 75% (12 fragments)	de Kok et al. (2014)

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Summary of common DNA assembly techniques in use.

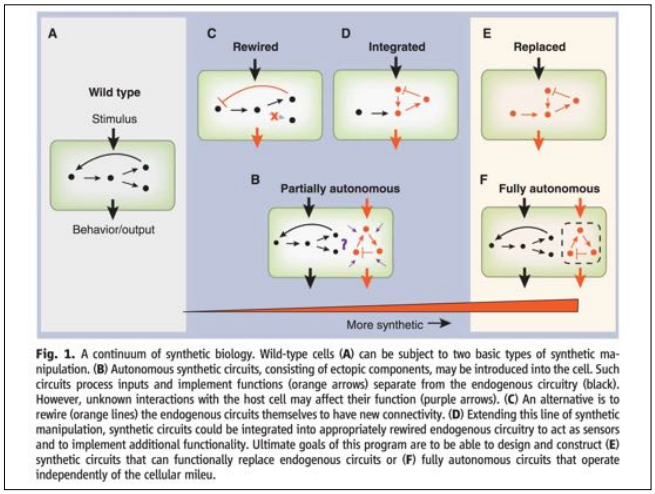

Fig. 1. A continuum of synthetic biology. Wild-type cells (**A**) can be subject to two basic types of synthetic manipulation. (**B**) Autonomous synthetic circuits, consisting of ectopic components, may be introduced into the cell. Such circuits process inputs and implement functions (orange arrows) separate from the endogenous circuitry (black). However, unknown interactions with the host cell may affect their function (purple arrows). (**C**) An alternative is to rewire (orange lines) the endogenous circuits themselves to have new connectivity. (**D**) Extending this line of synthetic manipulation, synthetic circuits could be integrated into appropriately rewired endogenous circuitry to act as sensors and to implement additional functionality. Ultimate goals of this program are to be able to design and construct (**E**) synthetic circuits that can functionally replace endogenous circuits or (**F**) fully autonomous circuits that operate independently of the cellular mileu.

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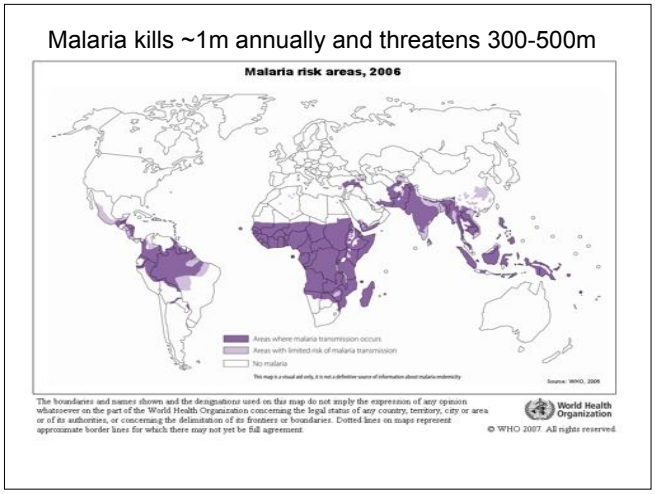
The new DNA assembly techniques allow the possibility of building large-scale synthetic genetic circuits. The field faces the next challenge of integrating synthetic circuits with existing regulatory systems. We'll look at an example where a plant metabolic network has been integrated into microbe.



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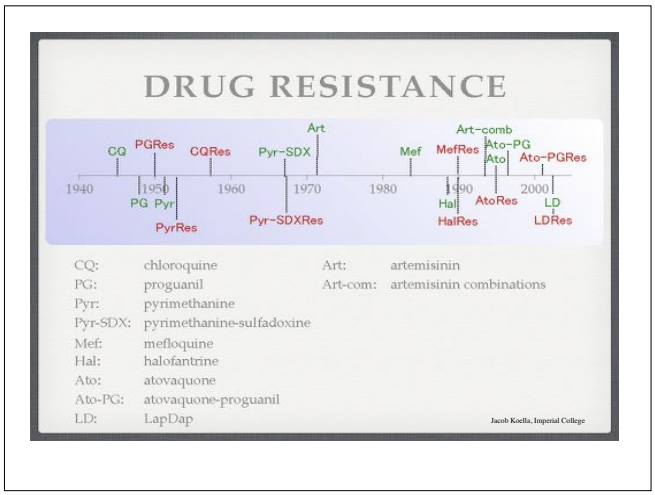
Mosquito borne malarial parasites have global distribution and affect millions.



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Resistance to malarial therapies occurs rapidly. A timeline shows the successive introduction of new therapies, as older therapies become less effective. The plant-derived drug artemisinin is a key component of modern antimalarial therapies.

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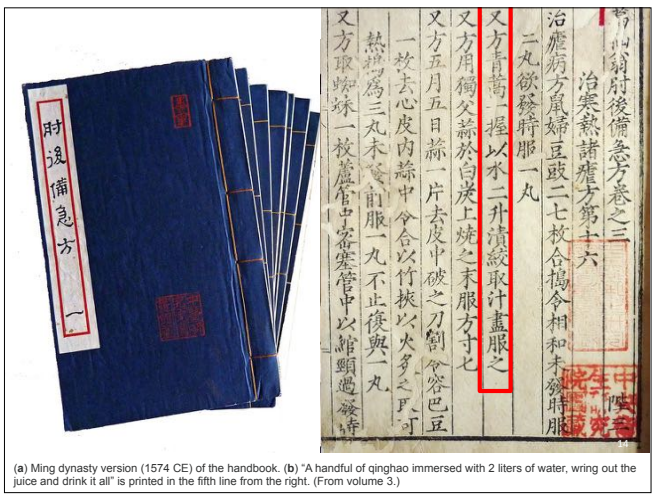
Chinese scientist Youyou Tu, who rediscovered the use of Artemisia extracts as an anti fever agent, and extracted the sesquiterpene artemisinin as an active anti-malarial drug.



(**a**) Ming dynasty version (1574 CE) of the handbook. (**b**) "A handful of qinghao immersed with 2 liters of water, wring out the juice and drink it all" is printed in the fifth line from the right. (From volume 3.)

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Key reference to production of active extracts from Artemisia (quinghao) plants from A Handbook of Prescriptions for Emergencies by Ge Hong (284–346 CE).

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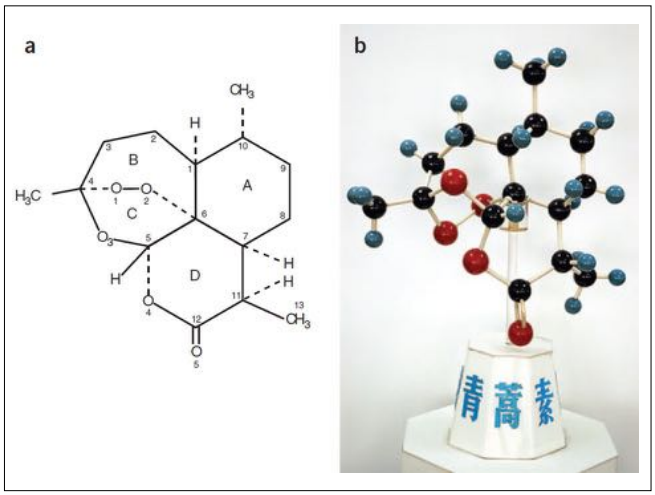
Image of a mature *Artemisia annua* plant, and ornamental shrub from the daisy family (Asteraceae).



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Artemisinin. (a) Molecular structure of artemisinin. (b) A three-dimensional model of artemisinin. Carbon atoms are represented by black balls, hydrogen atoms are blue and oxygen atoms are red. The Chinese characters underneath the model read Qinghaosu. The Chinese name for Artemisia is qinghao, and su means "basic element".

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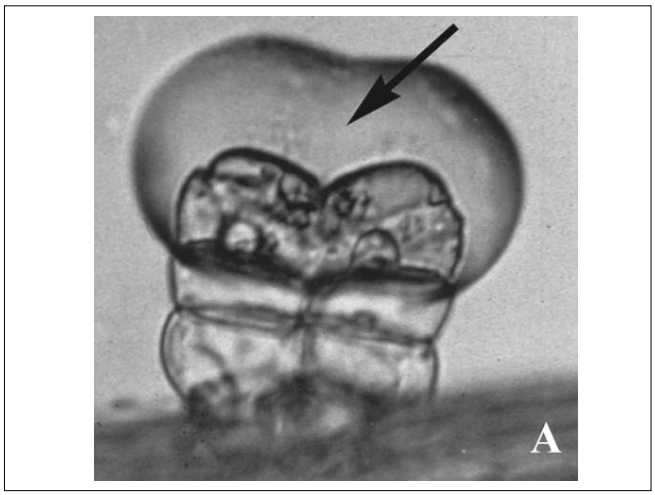
Scanning electron micrograph of the surface of an Artemisia leaf. The smaller box-shaped trichomes, or leaf hairs, are the major source of artemisinin in the plant.



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The trichomes consist of a multicellular column which is highly active in the biosynthesis of artemisin. This is capped by a subcuticular reservoir (arrowed) containing stored secondary compounds.

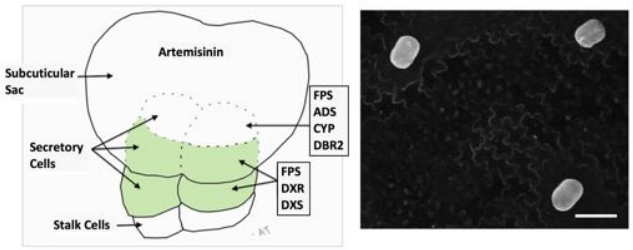

Figure 1.2: Schematic (left) and photo (right) of *Artemisia annua* trichomes. The trichomes on the right autofluoresce when visualized using UV fluorescence microscopy; bar = 100 µm. ADS, amorphadiene 4,11 synthase; CYP, cytochrome P 450 CYP71AV1; DBR2, double bond reductase 2; DXR, 1-deoxyxylulouse 5-phosphate reductoisomerase; DXS, 1-deoxyxylulose 5- phosphate synthase; FPS, farnesyl diphosphate synthase.

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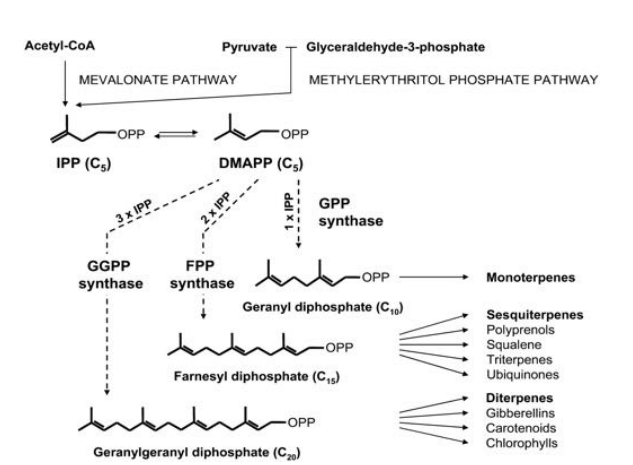
Localisation of biosynthetic enzymes in *Artemisia annua* trichomes. The column of cells express high levels of enzymes involved in the biosynthetic pathway. Products are secreted into the subcuticular sac. The cell complex behaves like a minature biofactory.



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Artemisin is a sesquiterpene, member of the terpenoid family. The different classes of terpenes are synthesised by addition of differnt numbers of isoprene "units" and decorated by modifying enzymes. Farnesyl diphosphate (FPP) is the immediate precursor to artemisinin production in the cell.

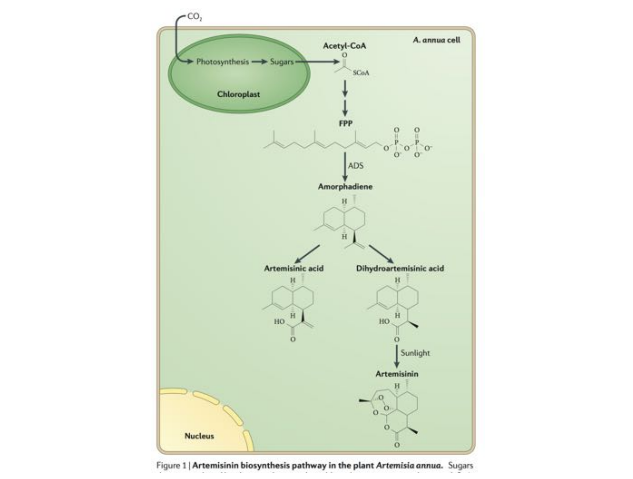

Figure 1 | **Artemisinin biosynthesis pathway in the plant *Artemisia annua*.** Sugars

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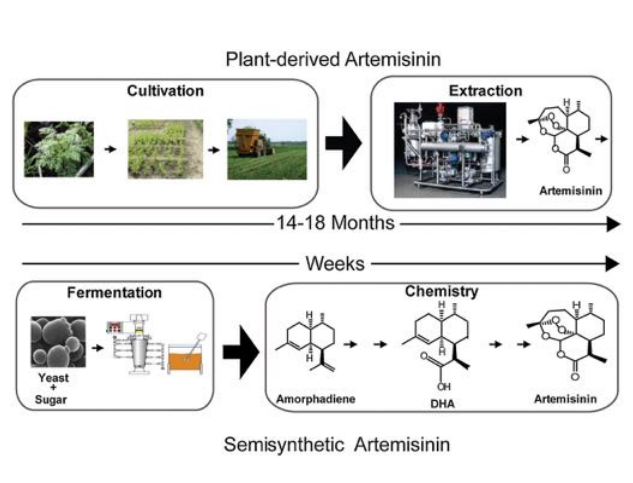
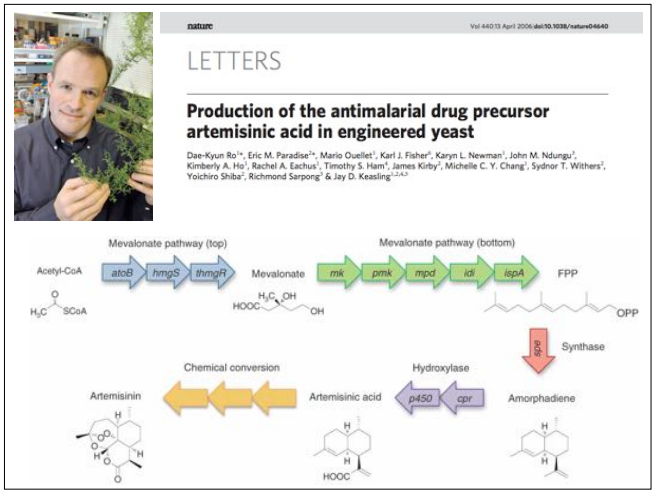
Pathway for enzyme catalysed conversion of FPP to artemisinin.



56

Artemisinin is naturally produced at low yields in slow-growing plants. Synthetic Biologists have taken up the challenge of transferring the artemisin pathway into yeast. This has potential benefits for lower cost and faster production of the drug.



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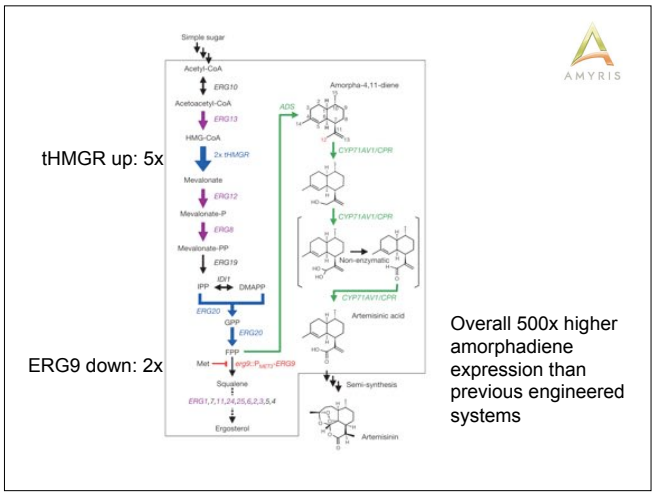
Assembly of a hybrid pathway for artemisin production in yeast.



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Schematic representation of the engineered artemisinic acid biosynthetic pathway in S. cerevisiae. Genes from the mevalonate pathway in S. cerevisiae that are directly upregulated are shown in blue; those that are indirectly upregulated by upc2-1 expression are in purple; and the red line denotes repression of ERG9 in strain EPY224. The pathway intermediates IPP, DMAPP and GPP are defined as isopentenyl pyrophosphate, dimethyl allyl pyrophosphate and geranyl pyrophosphate, respectively. Green arrows indicate the biochemical pathway leading from farnesyl pyrophosphate (FPP) to artemisinic acid, which was introduced into S. cerevisiae from A. annua. The three oxidation steps converting amorphadiene to artemisinic acid by CYP71AV1 and CPR are shown.

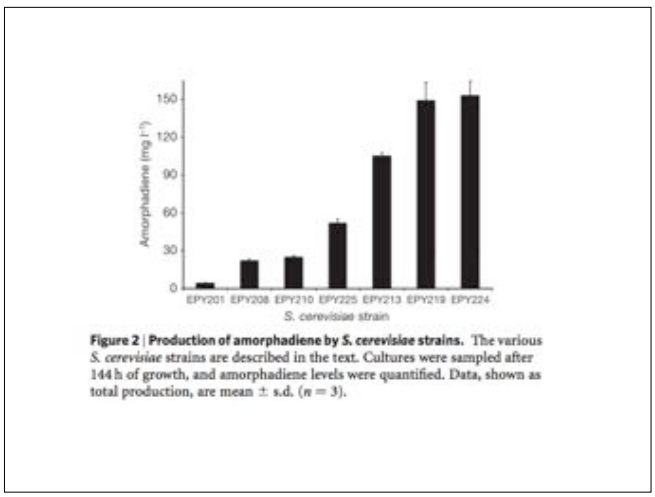

Figure 2 | Production of amorphadiene by *S. cerevisiae* strains. The various *S. cerevisiae* strains are described in the text. Cultures were sampled after 144 h of growth, and amorphadiene levels were quantified. Data, shown as total production, are mean ± s.d. ($n = 3$).

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Stepwise improvement of yields for amorphadiene in engineered yeast strains.

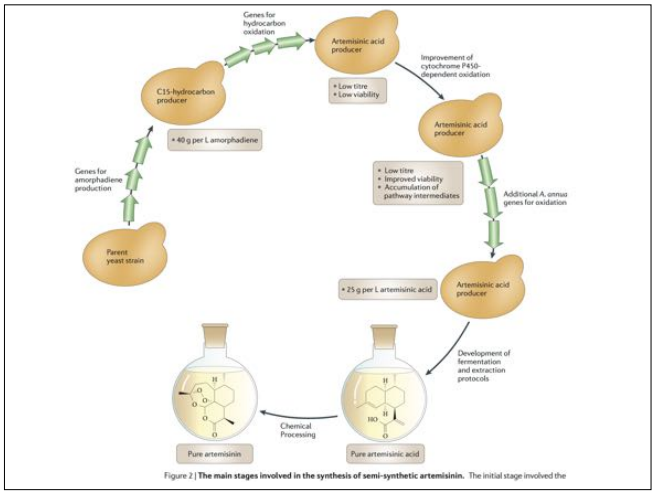

Figure 2 | **The main stages involved in the synthesis of semi-synthetic artemisinin.** The initial stage involved the

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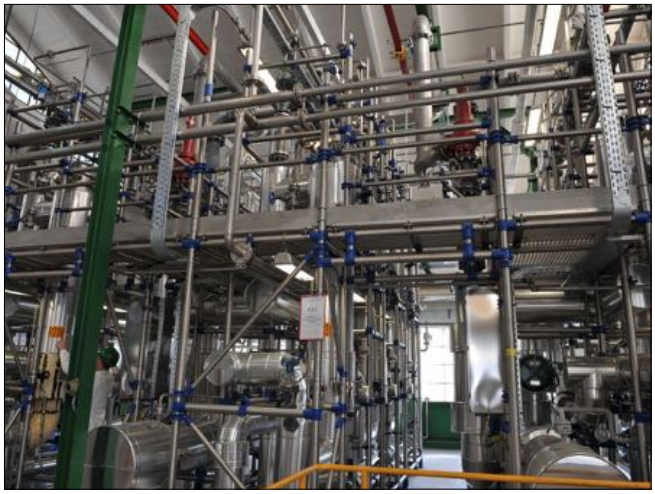
Representation of the main stages for improvement of production of semi-synthetic artemisin in yeast.



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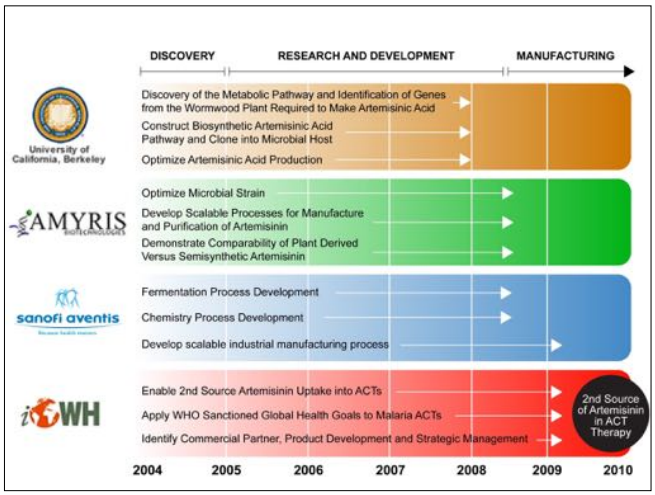
Commercial production facility for semi-synthetic artemisin, built by Sanofi in Italy.



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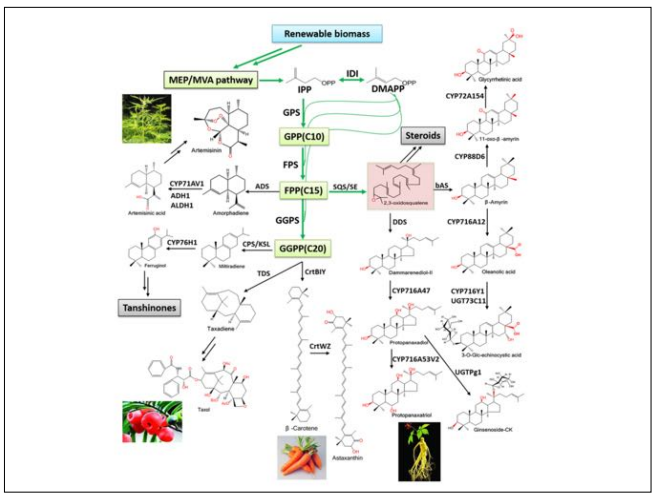
Cooperative efforts required for discovery, research and development and production.



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A wide variety of secondary compounds derived from plants are potential candidates for microbial production.

Lecture 1: Genetic modification in agriculture and the advent of Synthetic Biology.
Lecture 2: Genetic circuits and genome scale DNA engineering.

1. **Engineering principles for biology**
2. **Standardisation of DNA parts**
3. **Type IIS assembly and common syntax**
4. **Smart DNA registries and software tools**
5. **Chromosome engineering**
6. **Reprogramming metabolic networks in plants**
7. **Implementing plant pathways in microbes**

Lecture 3: Engineered logic and the control of gene expression.
Lecture 3: Self-organisation and reprogramming of multicellular systems.

Additional resources: **http://www.haseloff-lab.org** (Education)

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The next lecture will focus on the potential to build synthetic regulatory circuits.