Molecular diagnostics. L1
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Outline

- **Concept of Molecular Diagnostics**
- **History of Molecular Diagnostics**
- **Impact on Human Diseases**
- **Basis for Molecular Assay**
- **Management of the course**

The use of molecular biology techniques to expand scientific knowledge of the natural history of diseases, identify people who are at risk for acquiring specific diseases, monitor disease, determine appropriate treatment strategies, and predict disease outcomes.

The Molecular Biology Timeline

1865	Gregor Mendel, Law of Heredity
1869	Johann Miescher, Purification of DNA
1949	Sickle Cell Anemia Mutation
1953	Watson and Crick, Structure of DNA
1970	Recombinant DNA Technology
1977	DNA sequencing
1985	*In Vitro* Amplification of DNA (PCR)
2001	The Human Genome Project

Impact on Human Diseases: Novelty

Discovery of potential novel molecular markers of human diseases

Identification of novel molecular markers of human diseases

Utility of molecular markers to develop useful molecular assays for detection, diagnosis, and prediction of disease outcomes

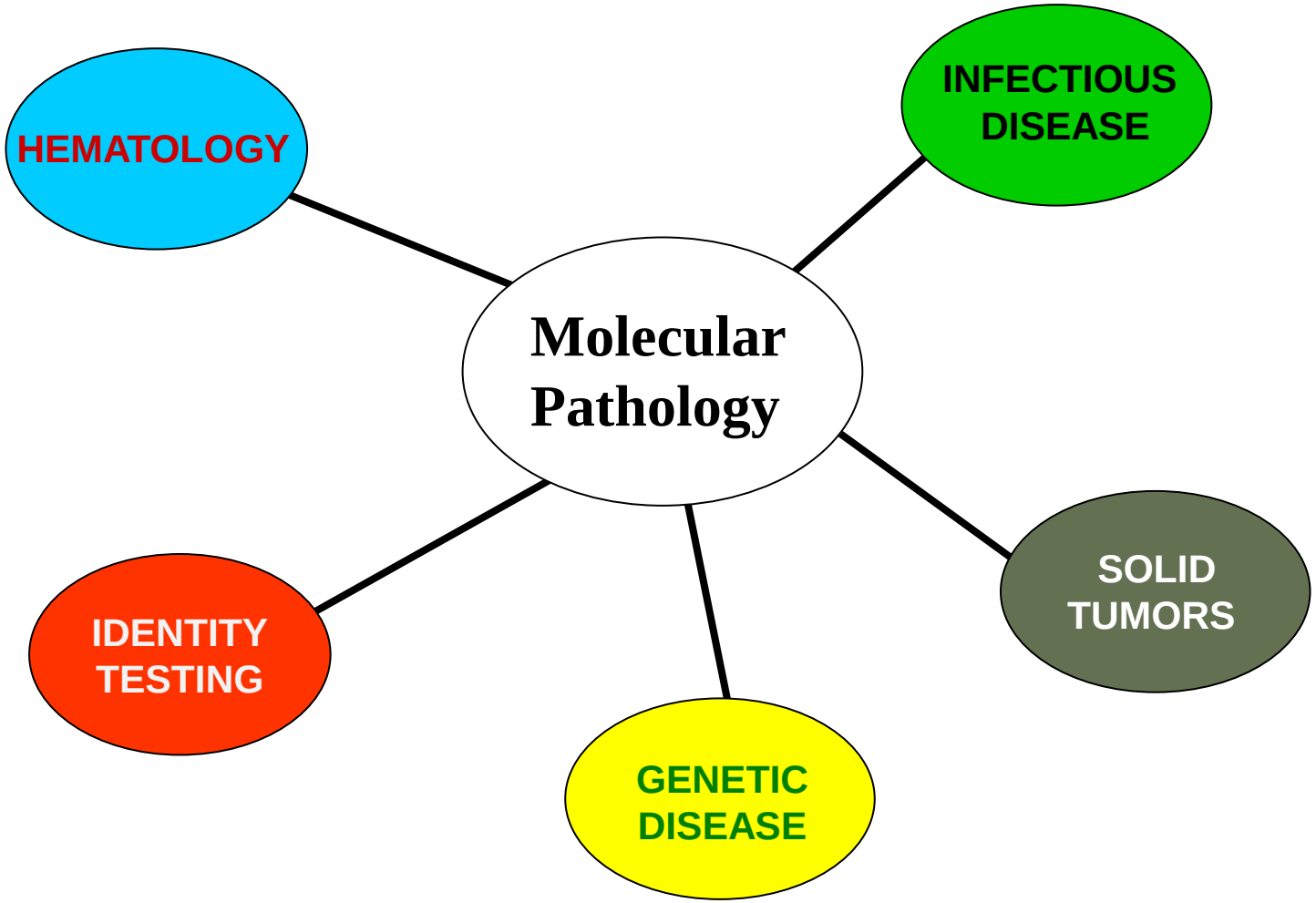
Impact on Human Diseases: Practical application

Diagnostic-Identity of a disease

Prognostic-Outcome of a disease

Predictive-Possibility of a disease

Therapeutic-Response of a disease to treatment


HEMATOLOGY
INFECTIOUS DISEASE
Molecular Pathology
IDENTITY TESTING
GENETIC DISEASE
SOLID TUMORS

Basis for Technology: Target specialty

Nucleic acids are targeted by molecular assays

- **Genetically-based diseases can be diagnosed**
- **Specificity can be controlled**
- **Single base changes can be detected**
- **Expression of gene product is not required**
- **Targets can be amplified $>10^5$**

Basis for Molecular Assays: Diseases

Cause (etiology)

Mechanism (pathogenesis)

Structural alterations (morphologic/molecular)

Functional consequences (clinical significance)

Basis for Molecular Assay: Pathogenesis

Understanding molecular pathogenesis of human disease enables effective utilization of molecular assays

Diagnostic

- **Distinguishing variants of human disease based on presence of specific molecular markers (chromosome translocations in Burkitt's lymphoma: *c-myc*)**

Basis for Molecular Assay: Pathogenesis

Understanding molecular pathogenesis of human disease enables effective utilization of molecular assays

Prognostic

- **Prediction of likely patient outcomes based on presence of specific molecular markers (gene mutations predicting clinical course in cancer)**

Basis for Molecular Assay: Pathogenesis

Understanding molecular pathogenesis of human disease enables effective utilization of molecular assays

Therapeutic

- **Prediction of response to specific therapies based on presence of specific molecular markers (gene mutations predicting poor drug sensitivity in lung cancer: *p53*, *k-ras*)**

Basis for Molecular Assay: Molecular biology

➢ Genetic Lesions in Human Disease

- **Identification of genetic markers**
- **Identification of disease-related genes**
- **Molecular targets for assay development**

Basis for Molecular Assay: Molecular biology

➢ Characterization of Gene Sequences

- **Facilitates characterization of disease-causing mutations**
- **Molecular targets for assay development**

Molecular Oncology

DIAGNOSTIC/PROGNOSTIC INFORMATION PROVIDED BY:

- Gross alterations in DNA content of tumors
- Cell cycle information
- Molecular Markers of Clonality
- Oncogene/Tumor Suppressor gene mutations
- Tumor Specific Translocations
- “Tissue specific” mRNA in tumor staging
- Minimal residual disease determination

Molecular Genetic Tests

Genetic test:

- Analysis of human
  - DNA
  - RNA
  - chromosomes
  - proteins
  - metabolites
- to detect heritable disease-related
  - genotype,
  - phenotype
  - karyotype
- for clinical purposes

Genetic Diagnosis

"Purpose"

Diagnostic Testing

Screening

Presymptomatic Testing

Prenatal testing

Preimplantation Diagnosis

Pharmacogenetic testing

Susceptibility to environmental agents

Genetic Alterations

Chromosomal alterations

“Gene-level” alterations

Test Choice

Cost

Sample requirements

Turnaround time

Sensitivity/Specificity

Positive/ Negative predictive value

Type of mutation detected

Genotyping vs mutation scanning

Conventional Cytogenetics
(Karyotyping)

Detect numerical structural chromosomal alterations

- trisomy
- monosomy
- duplications
- translocations, etc.

Conventional Cytogenetics
(Karyotyping)

evaluate all chromosomes

- *prior* specification of chromosome unnecessary
- detect unsuspected abnormality
- detect *balanced* alterations
  - (No gain or loss of genetic material)

FISH may be performed

- characterize unexpected alterations

Conventional Cytogenetics
(Karyotyping)

Disadvantages:

- Need for live cells to grow in culture
  - failure <1%*
- Turnaround time - up to 10 days
  - - 90% of results w/in 14 days*
- Labor Intensive

*http://www.neuro.unn.ru/sites/default/files/opredelenie_zhiznesposobnosti.pdf; https://bit.ly/3og0enM

FISH

Use of fluorescently labeled probes to specifically visualize

- entire chromosomes (chr. paint probes)
- centromeres (centromeric probes)
- specific loci (locus-specific probes)

Metaphase

- All types of probes

Interphase

- Centromeric and locus-specific probes only

FISH

Identify:

- translocations
- marker chromosomes
- Small deletions/duplications w/ locus-specific probes
  - e.g., DiGeorge's syndrome

Interphase FISH

rapid (<48 hours) detection of

- Numerical abnormalities
- Duplications/deletions/amplifications
- translocations
- mosaicism

Interphase FISH

Prenatal Chr.13, 18, 21, X + Y

- approx. 75-85% of all clinically relevant abnormalities

Dual color FISH w/subtelomeric probes:

- Prenatal dx of chromosomal translocations

Interphase FISH

Need for confirmatory conventional cytogenetic testing

Need to specify chromosome

- Information only about specific chromosome/locus tested

Metaphase FISH

Supplement conventional cytogenetics

- Identify marker chromosomes
- extra unknown material attached to chromosome/loss of segment
- detect/identify rearrangements (incl. cryptic translocations)
- identify/quantify mosaicism

Metaphase FISH

Need to specify chromosome/locus

- Multiple tests to identify marker chromosome
- Multiprobe FISH

Gene Dosage

Gains/Losses

Comparative genomic hybridization (CGH)

- Label normal and test DNA with separate dyes
- competitively hybridize to
  - Metaphase Spread or
  - cDNA array
- Detect Gains and losses

Gene Dosage

Gains/Losses

Classical CGH

- Hybridize to metaphase spread
  - Resolution approximately 5Mb
- Information on *all* chromosomes
- No need for culture
  - can use archival material (e.g., placenta, tumor, etc.)
- Single cell DNA amplification & CGH
  - applicable to preimplantation genetic diagnosis (PGD)

Gene Dosage
Gains/Losses

Array-based CGH

- hybridize to BAC-based or cDNA array
- Higher resolution (50kb vs 5MB)

Gene Dosage
Gains/Losses

PCR-based methods

- Real-time (quantitative) PCR
- microsatellite PCR
- Long-range PCR
- probe amplification techniques

Rapid

For *specific* loci

- May be “multiplexed” for multiple loci

Molecular Tests

Test for:

- karyotype
- gain or loss of genetic material (“dosage”)
- genetic linkage
- known/recurrent mutations
- variations in lengths of repeat sequences
- alterations in DNA methylation
- unknown mutations in multiple genetic segments

Types of mutations-gene

Point mutations
- Missense (change an amino acid)
- Nonsense (premature termination)
- Silent

Deletion
- Large variation in size

Insertion

Duplication

Splice site

Regulatory

Expanded repeat

Missense mutations

When is a missense mutation significant?

- known structural and functional domain
- evolutionarily conserved residue
- independent occurrence in unrelated patients
- absent in large control sample
- novel appearance & cosegregation w/disease phenotype in pedigree
- *In vitro* loss of function
- restoration of function by WT protein

Deletions

Complete/partial gene deletion

- Duchenne Muscular Dystrophy
- Alpha thalassemia

Multiple genes “contiguous gene syndromes”

- DiGeorge Syndrome
- TSC2-PKD1
- WAGR syndrome

Insertions

Tay Sachs Disease

- 4bp insertion in Ashkenazi Jews

Hemophilia A

- L1 insertion in FVIII gene (1% of patients)

Other mutations

Cap-site mutants

Mutations in initiation codons

Creation of a new initiation codon

Mutations in termination codons

Polyadenylation/cleavage signal mutations

Mutation Testing

Tests for recurrent mutations

- Limited number of specific mutations
  - significant proportion of cases e.g., Factor V Leiden, Hemochromatosis

Mutation Scanning Methods

- Multiple “private” mutations of one or more genes
  - e.g., *BMPR2* mutations in familial primary pulmonary hypertension (PPH)

Combination

- e.g., *BRCA1/2*, *CFTR*, etc.

Recurrent Mutation Tests

Many rapid methods

High sensitivity/specificity

Test choice - laboratory preference

- Workflow, equipment, kit availability
- patent issues, etc.

Detect

- heterozygotes,
- compound heterozygotes
- homozygotes

Recurrent Mutation Tests

Choice of mutation tested:

- Clinical syndrome
- Family history
- Ethnicity

Positive results:

- Unambiguous
- Technical false positive rare (*most* methods)
- Positive predictive value, penetrance, etc.

Recurrent Mutation Tests

Negative predictive value:

- Population screening:
  - 1- (ethnic prevalence x [1 - sensitivity for specific ethnic group])
- Family history (index case w/ unknown mut)
  - 1 - (prior probability x [1- sensitivity for specific ethnic group])
- Family history (known mutation in index case)
  - 100%
- Affected individual (unknown mutation)
  - 0%

Recurrent Mutations

Methods

- PCR-RFLP
- Allele-specific probes/primers
- Direct sequencing/“Minisequencing”/ Pyrosequencing
- Molecular Beacons/TaqMan probes
- Oligonucleotide ligation assay
- Mass spectroscopy-based methods

Mutation Scanning Methods

Test one or more genes for unknown variation in

- Exons
- Introns
- splice sites
- Promoters/enhancers
- “locus control region”

Screening methods

- Sensitivity determined by specific mutation
- Need for multiple conditions
- *One* datapoint per gene segment evaluated
- Screen for *presence,* not *identity* of mutation

Mutation Scanning Methods

Direct Sequencing

- Screen presence *and* identity of mutation
- Bidirectional sequencing
- 2 data-points *per base* sequenced
- DNA sequencing
  - usu. multiple exons tested
  - splice-site mutations may be missed, especially mutations deep in large introns
- RNA sequencing
  - need for cells w/c express gene
  - “nonsense mediated decay”
  - RNA more labile

Direct Sequencing Methods

Automated fluorescent sequencing

- DNA/cDNA amplification, purification, and re-amplification with Fluorescent "Big-Dye" terminators
- widely available
- need to visually scan electropherograms
  - verify "base calling", heterozygous bases

Direct Sequencing Methods

Pyrosequencing

- limited to short sequences
- need to optimize algorithm for each segment

Chip-based sequencing

- rapid
- reduced sensitivity for heterozygous and frame-shift mutations

Interpretation of Variant

Previously reported variant

- Known to be cause of disorder
- Known to be “neutral variation”

Interpretation of Variant

New variant:

- Type likely to be associated w/disorder
  - frame-shift mutation
  - start “ATG” mutation
  - “Stop codon”
  - splice-junction mutation
  - non-conservative missense in active site

Genetic testing additional considerations:

Benefits Vs. Risk of Testing:

- Availability of treatment/prevention of clinical syndrome
- Presence or absence of pre-clinical manifestations
- Discrimination:
  - Insurance
  - Employment
  - Confidentiality

Additional Considerations

Screening vs testing “index” case

Index case
- Known disease;
- negative result:
  - mutation not detected
  - carrier testing not possible

Locus heterogeneity:
- Long QT, red-cell membrane defects, phenylketonuria, etc.

Variable “penetrance”
- variable predictive value of positive results

Variable expressivity

Additional Considerations

Potential interventions:

- Behavioral
  - lung cancer-risk - smoking cessation;
  - heart disease risk - diet/exercise;
  - risk of breast/colon cancer - screening acceptance
- Medical
  - e.g., prophylactic mastectomy/thyroidectomy;
  - blood-letting/blood donation;
  - Antiarrhythmics, etc.

Additional Considerations

Pre-morbid/clinical syndrome

- Is there a clinically identifiable syndrome?
- ? Need for intervention *prior* to clinical manifestations

Technical considerations

- e.g., Fragile X-syndrome

Patent issues

- affect availability/cost of testing

Factors affecting utility of genetic testing

Increased Utility

- High morbidity and mortality of the disease
- Effective but imperfect treatment
- High predictive power of genetic test (high penetrance)
- High cost or onerous nature of screening and surveillance methods
- Preventive measures that are expensive or associated with adverse effects

Decreased utility

- Low morbidity and mortality of disease
- Highly effective and acceptable treatment (i.e., no harm is done by waiting for clinical disease to treat patient)
- Poor predictive power of the genetic test (low penetrance)
- Availability of inexpensive, acceptable, and effective surveillance methods (or need for surveillance whether or not one has increased genetic risk)
- Preventive measures that are inexpensive, efficacious, and highly acceptable - e.g., folate supplementation

Ordering Molecular Tests

Patient preparation: None

- **Avoid heparin**, it interferes with PCR

Specimens:

- Fresh whole blood: EDTA/Citrate
- Fresh tissues
- Frozen tissues
- Paraffin embedded tissues
- Slides etc.

Ordering Molecular Tests

Specimen Handling

DNA-based tests:

- Room temperature, up to 72 hours (maybe more with special buffers)

RNA-based tests:

- Deliver ASAP (4-6 hours)
- Special considerations for proprietary test.

Ordering Molecular Tests

Essential info (Molecular Genetic Tests):

- Clinical information
- pedigree, if possible
- Race
- reason for testing

Informed consent:

- Nature of test; availability of genetic counseling; implications of positive and negative tests, etc.

Current Techniques Applied to Molecular Pathology (one gene – one disease)

Southern blot

Dot blot/Reverse dot blot

Polymerase chain reaction

SSCP/DGGE

RT-PCR

DNA sequencing

TaqMan, real-time PCR

Invader assay

In situ hybridization

New Techniques Coming to Molecular Pathology *(all genes – all diseases)*

Microarray hybridization

High-density microarray hybridization

Array comparative genomic hybridization

Whole-genome sequencing

Classes of Novel/Unexpected Sequence Variants Identified by Whole Genome Sequencing

Missense variants of uncertain significance in known gene

Variants and deleterious mutations in unknown gene(s)

Deleterious mutations in unintended target (e.g., BRCA mutations in a baby)

Ethical Dilemmas of Whole Genome Sequencing

Revelation of “off-target” mutations

Many revealed disorders will have no prevention or treatment

Revelation of nonpaternity, consanguinity, incest

Costs of genetic counseling and follow-up

Possible forensic uses of data

Data storage and privacy

Huge number of novel missense variants

Conclusion

What's So Great About Molecular Diagnostics?

- As many as 5,000 diseases have direct genetic causes
- High sensitivity and increased specificity for most tests adds diagnostic utility
- Potential for simple standardized procedures an automation
- rapid throughput
- Increased number of techniques for infectious diseases and tumor diagnostics
- A viable reflex for equivocal morphology
- Prices are falling

Conclusion

The main goal of the molecular diagnostics is to provide molecular information that will combine with and complement information related to patient history and symptomology, clinical laboratory results, histopathological findings, and other diagnostic information to provide a more sensitive, precise, and accurate determination of disease diagnosis and/or guidance toward appropriate and effective treatment options.

Youtube videos screened

What is newborn screening Animated video for parents

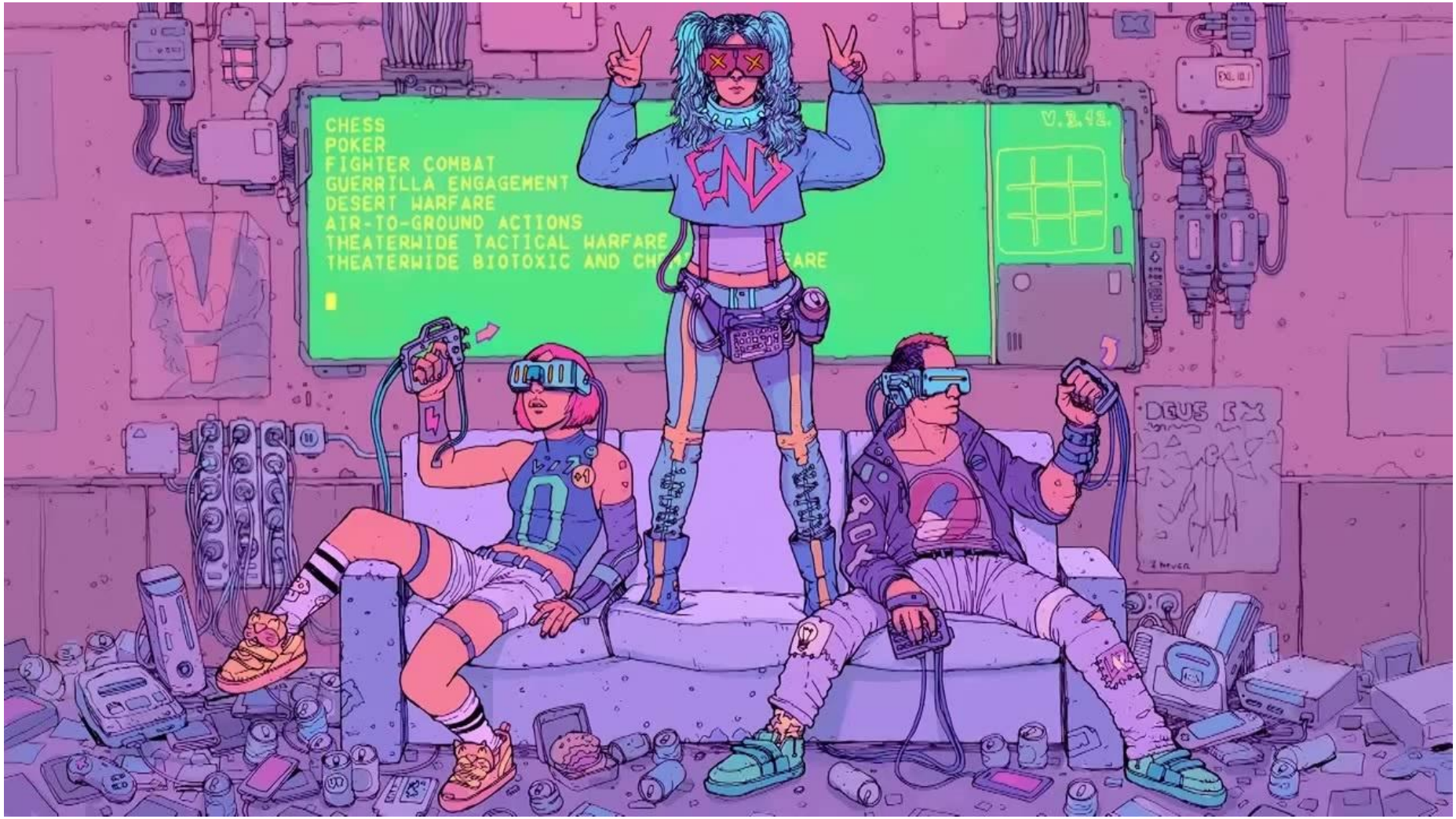
The Evolution of PCR

Molecular machines win Nobel Prize

QUESTIONS OR COMMENTS?

To read:

https://www.nature.com/articles/s41579-021-00598-5;
https://www.intechopen.com/online-first/75013;
https://onlinelibrary.wiley.com/doi/10.1111/nan.12716;
https://www.genome.gov/human-genome-project;
https://bit.ly/3koBNmW;
https://www.sciencedaily.com/releases/2021/09/210914111232.htm;
https://www.bmj.com/content/375/bmj-2021-066288;
https://www.frontiersin.org/articles/10.3389/fmed.2021.737602/full;
https://bit.ly/3bX5VkM;
https://bit.ly/30bWdIY;
https://www.frontiersin.org/articles/10.3389/fgene.2021.720507/full;
https://www.nature.com/subjects/cytogenetics
https://bit.ly/3BZW0FM
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7903223/;
https://www.sciencedirect.com/science/article/pii/S1046202320300591;
https://www.nature.com/subjects/disease-genetics;
https://www.sciencedaily.com/releases/2021/08/210826170151.htm;
https://www.nature.com/articles/s41467-021-22444-1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