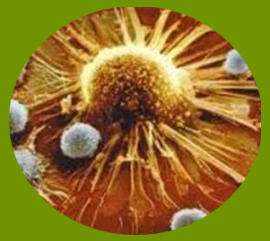
Cryphonectria parasitica tendrils on chestnut tree bark (Photo: Ministry of Agriculture and Regional Development Archive, Ministry of Agriculture and Regional Development, Bugwood.org)



6/22/2014

Spectrophotometry and Beer's Law

B3 Summer Science Camp at Olympic High School

Dr. Jennifer Weller

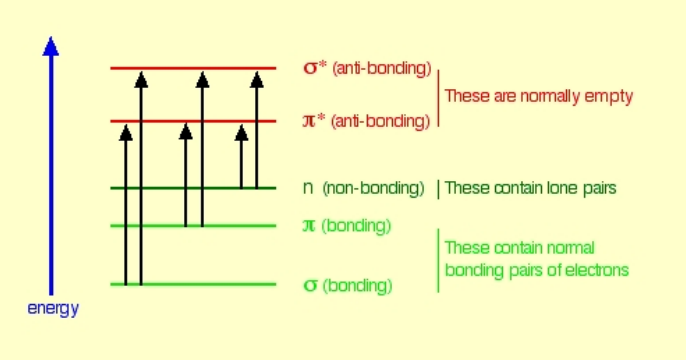
Spectroscopy measures how matter interacts with radiation.

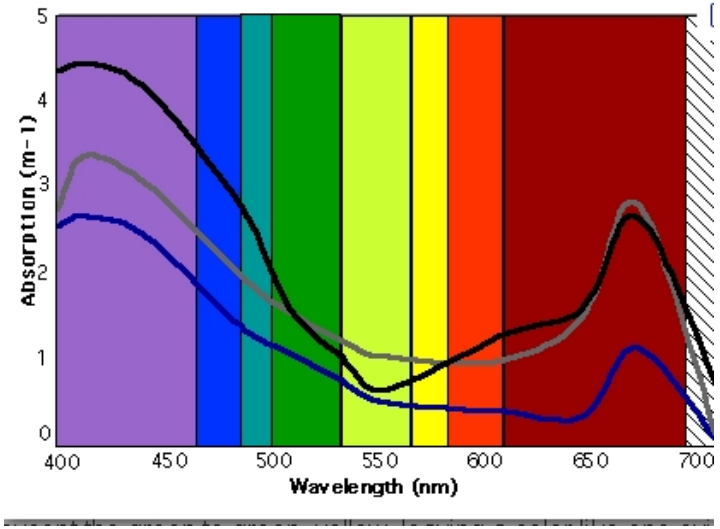
$$E = h\nu \qquad \lambda = \frac{c}{\nu}$$

- Radiation is characterized by frequency or wavelength.
  - Radiation passes through or bounces off matter resulting in a change in the number of photons (intensity), a frequency shift or a scatter pattern
- Absorbance
  - Interactions of photons with matter are frequency/wavelength dependent so monochromatic light (single-wavelength) is used.
  - The amount of light before it gets to your material (incident intensity) is decreased by the amount the material absorbs, so exiting light intensity is less than *incident* light intensity.
- Scattering
  - Photons *reflect* from the material – coherent light will change direction
- Fluorescence
  - Interactions with molecules causes photons to shift to higher electron orbitals, energy is lost by photons as they decay back down

Absorbance: when light is *absorbed* by molecules the energy of the photon may promote an electron from a ground state to a new orbital.

- The energy provided has to match the energy difference in the orbitals, changing the state of the molecule.
  - This energy can be released by electron decay to the original state or a new state, with photon release (light is emitted at a new wavelength), or by transfer to another molecule or dissipation to the environment.
  - For proteins and nucleic acids, photons in the UV and near-UV/visible range have the right energy to be absorbed.
- Organic molecules subjected to UV-visible light have transitions from
  - pi bonding to pi antibonding orbitals
  - non-bonding to pi antibonding orbitals
  - non-bonding to sigma anti-bonding orbitals.





Beer-Lambert Law

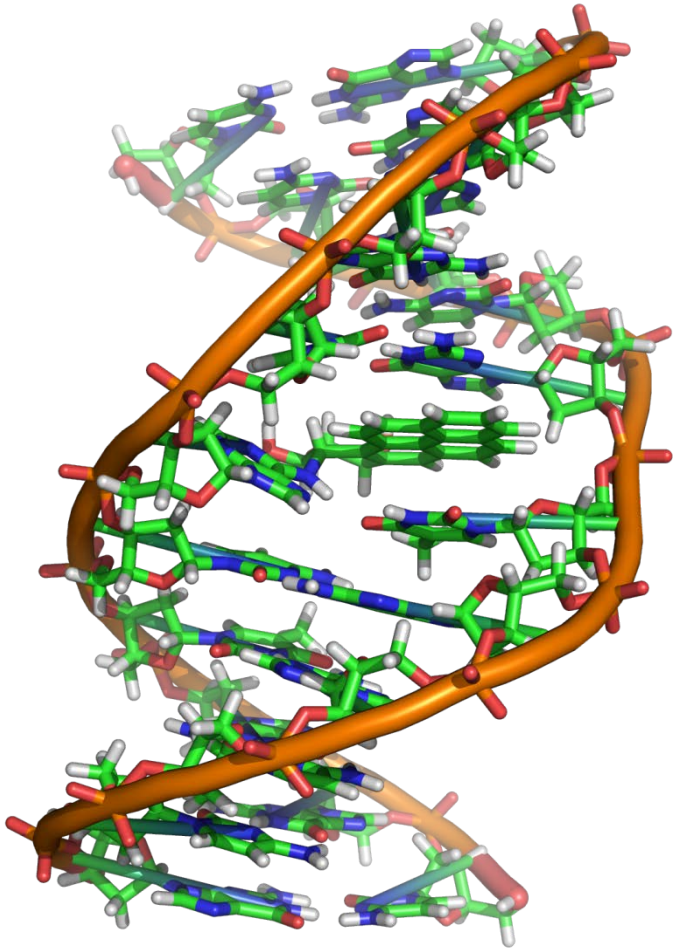
- The law is an expression of a relationship:

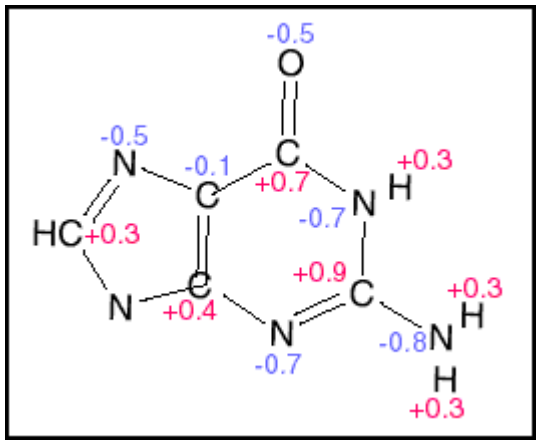
$$A = \varepsilon c \ell$$

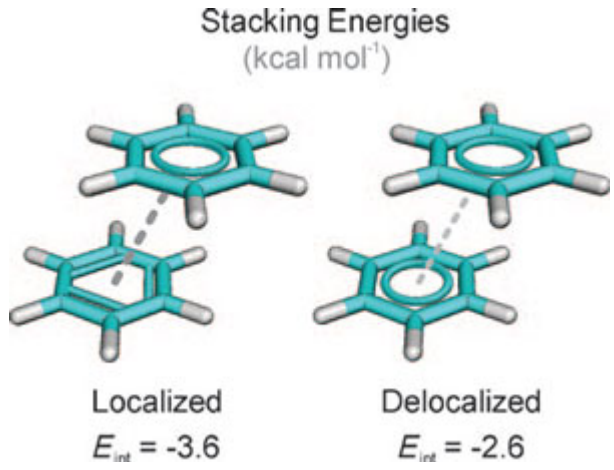
  - A is the absorbance
  - ε is a constant for a given substance, if it is molar units them it is {Liters per mole per centimeter, or L- mol $^{-1}$ cm^{-1}}
    - For nucleic acids ε_{260} = 50 ml/ng-cm for dsDNA, 40 ml/ng-cm for RNA and 33 ml/ng-cm for ss DNA.
  - c is the concentration (units are in moles/Liter)
  - ℓ is the path ℓength that the light passes through the sample (converted to centimeters for whatever spectrophotometer you used, so units of the constant cancel out properly)

Absorbance spectrum

- Biopolymers are complicated,

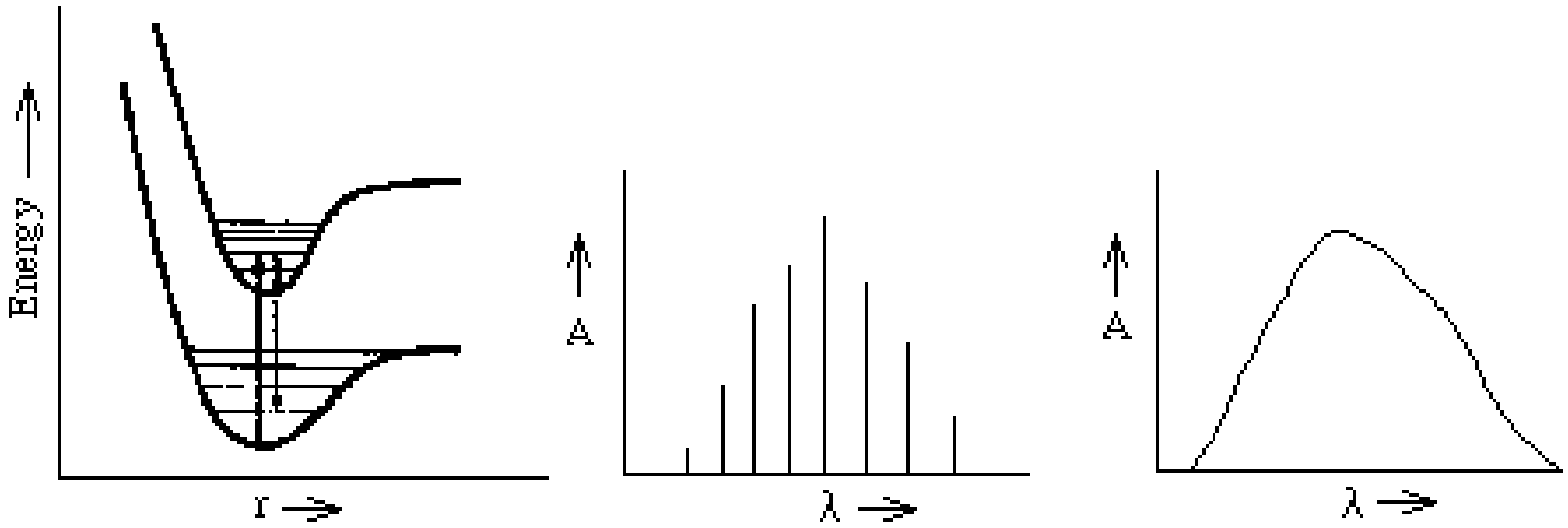






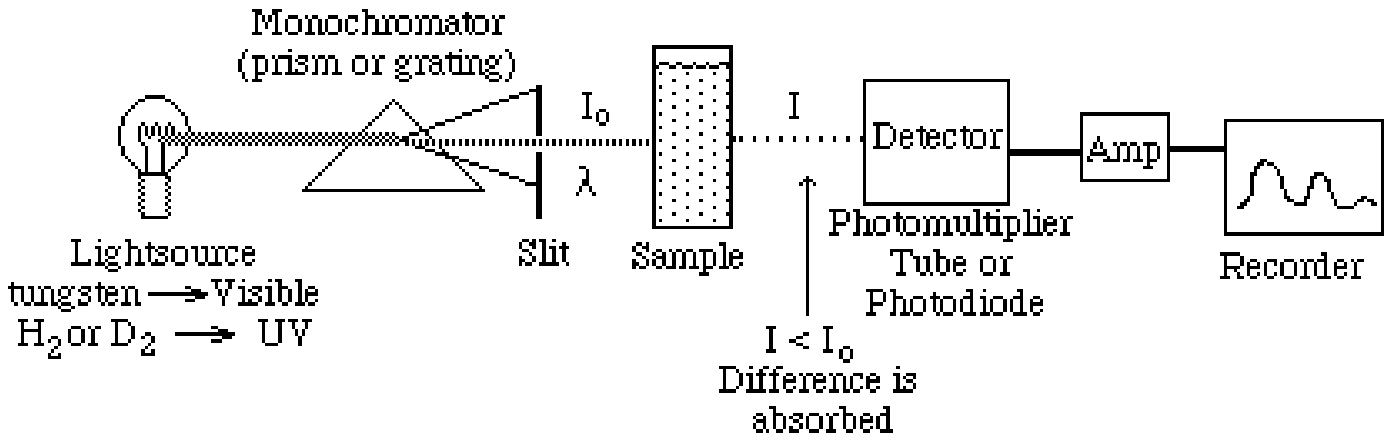
Absorbance spectrum

- Biopolymers have many vibrational energy levels so there are many closely-spaced individual absorption peaks that sum to a broad peak.



Measuring the changes in light as it interacts with matter is called spectrophotometry

- A spectrophotometer selects light of a determined wavelength from a source, passes it through a sample, and detects the number of photons (intensity) and/or frequency of the wavelength that reaches the detector.



Absorption of light by DNA and Proteins

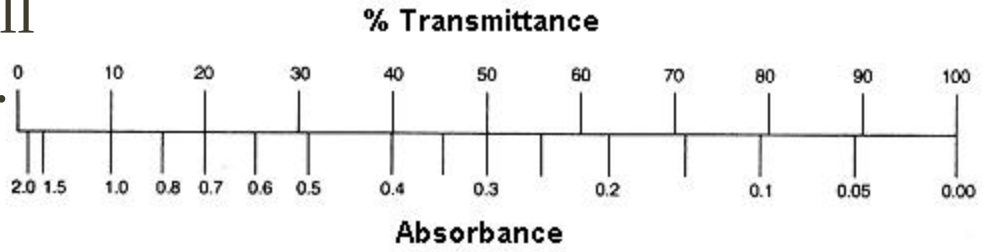
- DNA and proteins absorb light (photons) whose frequency is in the ultraviolet range (240-300nm).
- If some photons are absorbed going through a sample, fewer will emerge from the far side of a sample
- The intensity of the light (number of photons) will decrease on the far side.

Transmittance, $T = P / P_0$

% Transmittance, $\%T = 100\ T$

Absorbance,

$$A = \log_{10} P_0 / P$$
$$A = \log_{10} 1 / T$$
$$A = \log_{10} 100 / \%T$$
$$A = 2 - \log_{10} \%T$$



The law says that the fraction of light absorbed by each layer of solution is the same.

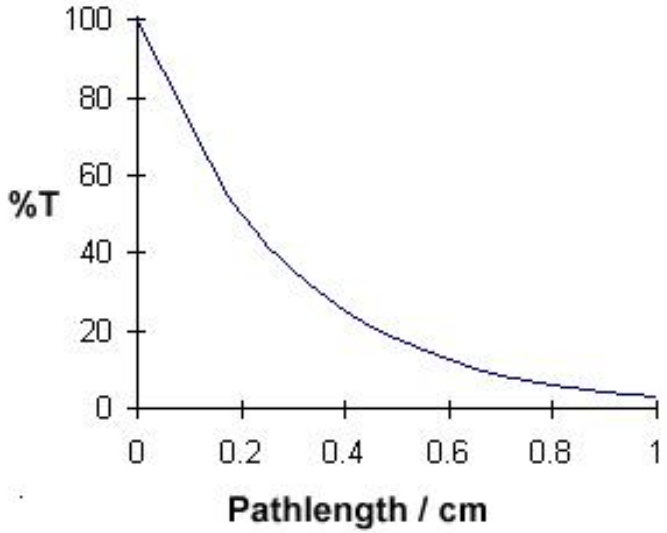
- Say that the fraction is 0.5 for each 0.2cm layer.
- Here is the data:

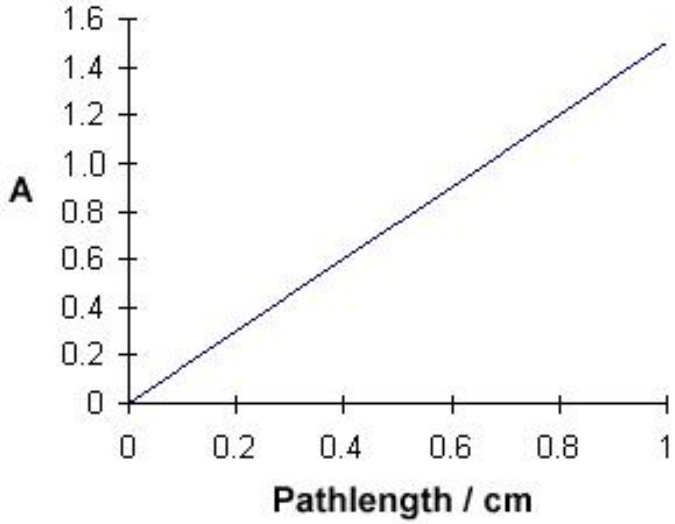
Path length / cm	0	0.2	0.4	0.6	0.8	1.0
%T	100	50	25	12.5	6.25	3.125
Absorbance	0	0.3	0.6	0.9	1.2	1.5

Graphing the relationship.

$$A = \varepsilon c \ell$$

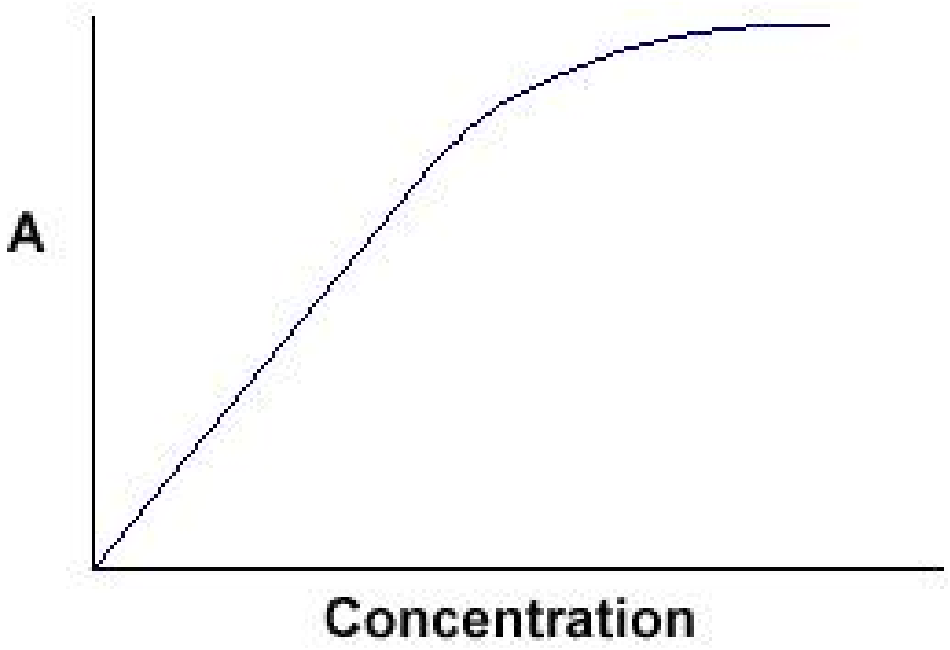
$$\%T = 100(P/P_0) = e^{-\varepsilon c l}$$





Is the law true everywhere?

- When the solution is too concentrated no light at all passes through.

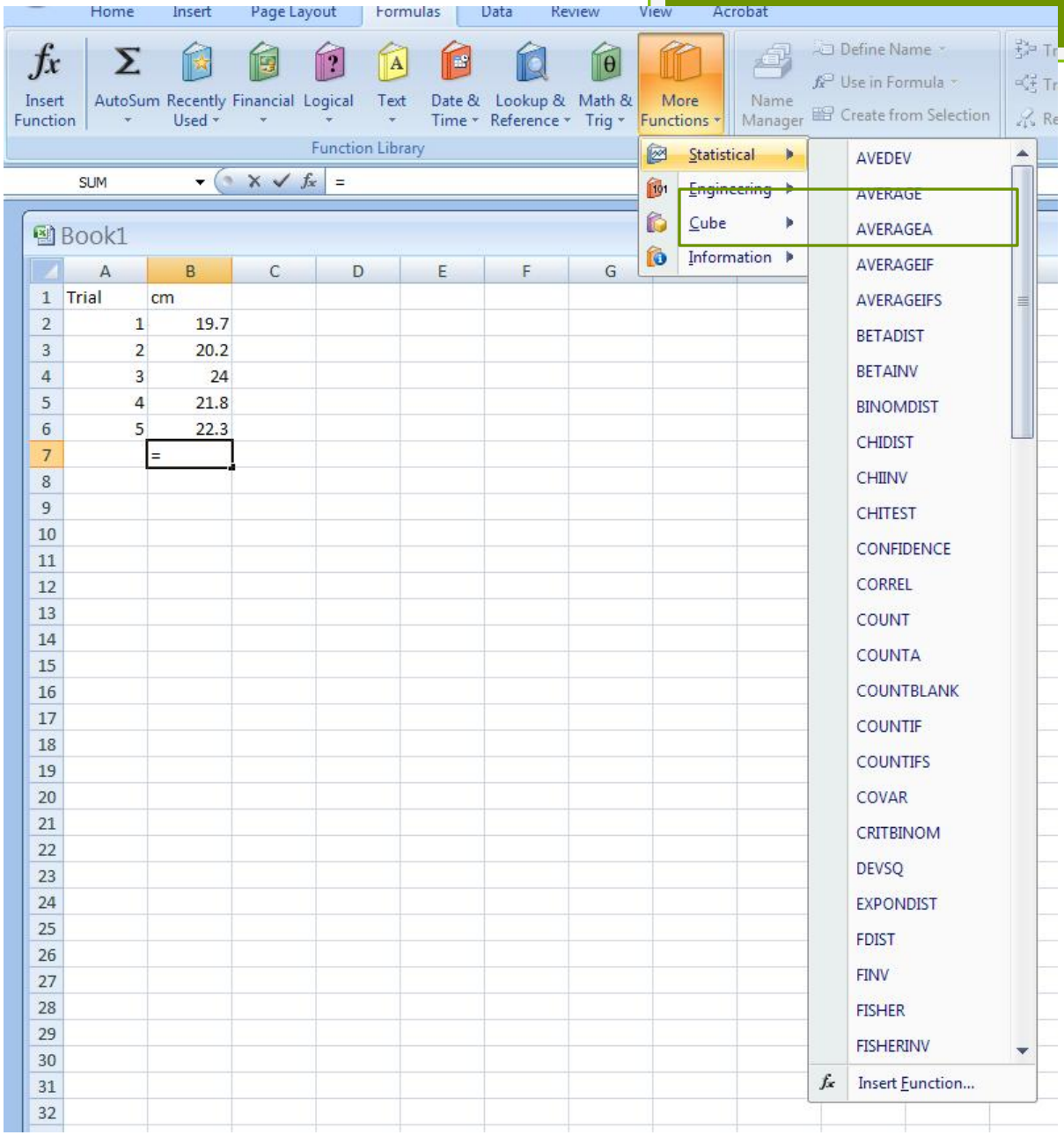


Averages of Measurements

- On the spectrophotometer you took multiple readings.
- Usually you take the mean or median in order to get the best *estimate* of accuracy.
  - The mean: add the replicate measurements together and divide by the number of measurements you took of that sample.
  - The median: take the middle value of the measurements (separating the top half and the bottom half – this does not work if you only took 2 replicates)

Finding the *range* of your measurements

- The smallest interval that contains all your data is the range (the highest to lowest value)
  - The variance shows how far the numbers are from the average (mean).
  - You calculate variance by subtracting the mean from each measurement and taking the square , summing these values and dividing by the number of values.
- The standard deviation is often used – it is the *square root* of the variance
  - That way it has the same units as the measurements.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CHIDIST
CHIINV
CHITEST
CONFIDENCE
CORREL
COUNT
COUNTA
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COUNTIF
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COVAR
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EXPONDIST
FDIST
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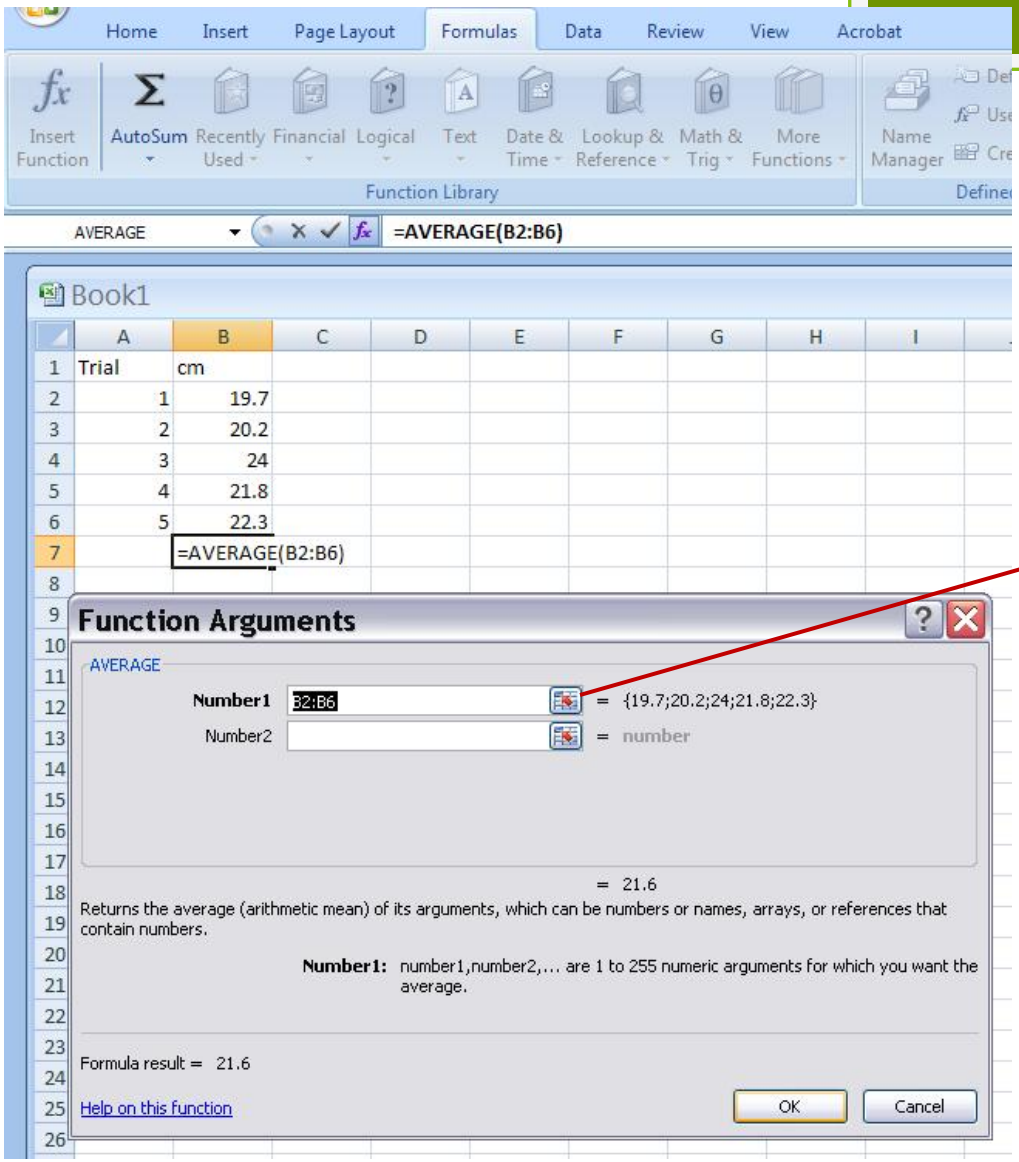
1 19.7
2 20.2
3 24
4 21.8
5 22.3
=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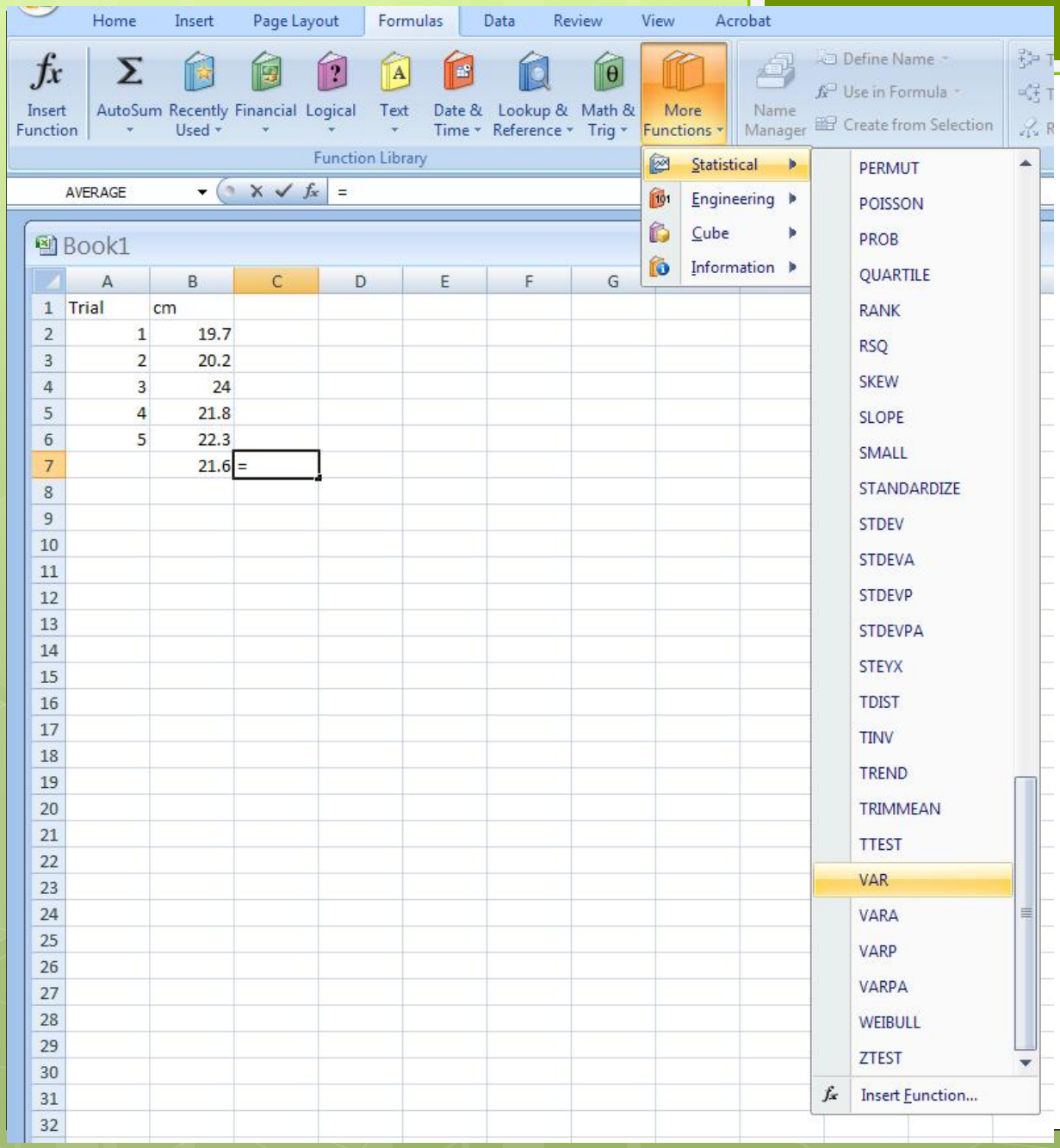
AVERAGE
=AVERAGE(B2:B6)
Book1
Trial
cm
1 19.7
2 20.2
3 24
4 21.8
5 22.3
=AVERAGE(B2:B6)
Function Arguments
AVERAGE
Number1
B2:B6
= {19.7;20.2;24;21.8;22.3}
Number2
= number
= 21.6
Returns the average (arithmetic mean) of its arguments, which can be numbers or names, arrays, or references that contain numbers.
Number1: number1,number2,... are 1 to 255 numeric arguments for which you want the average.
Formula result = 21.6
Help on this function
OK
Cancel

B2:B6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Information
PERMUT
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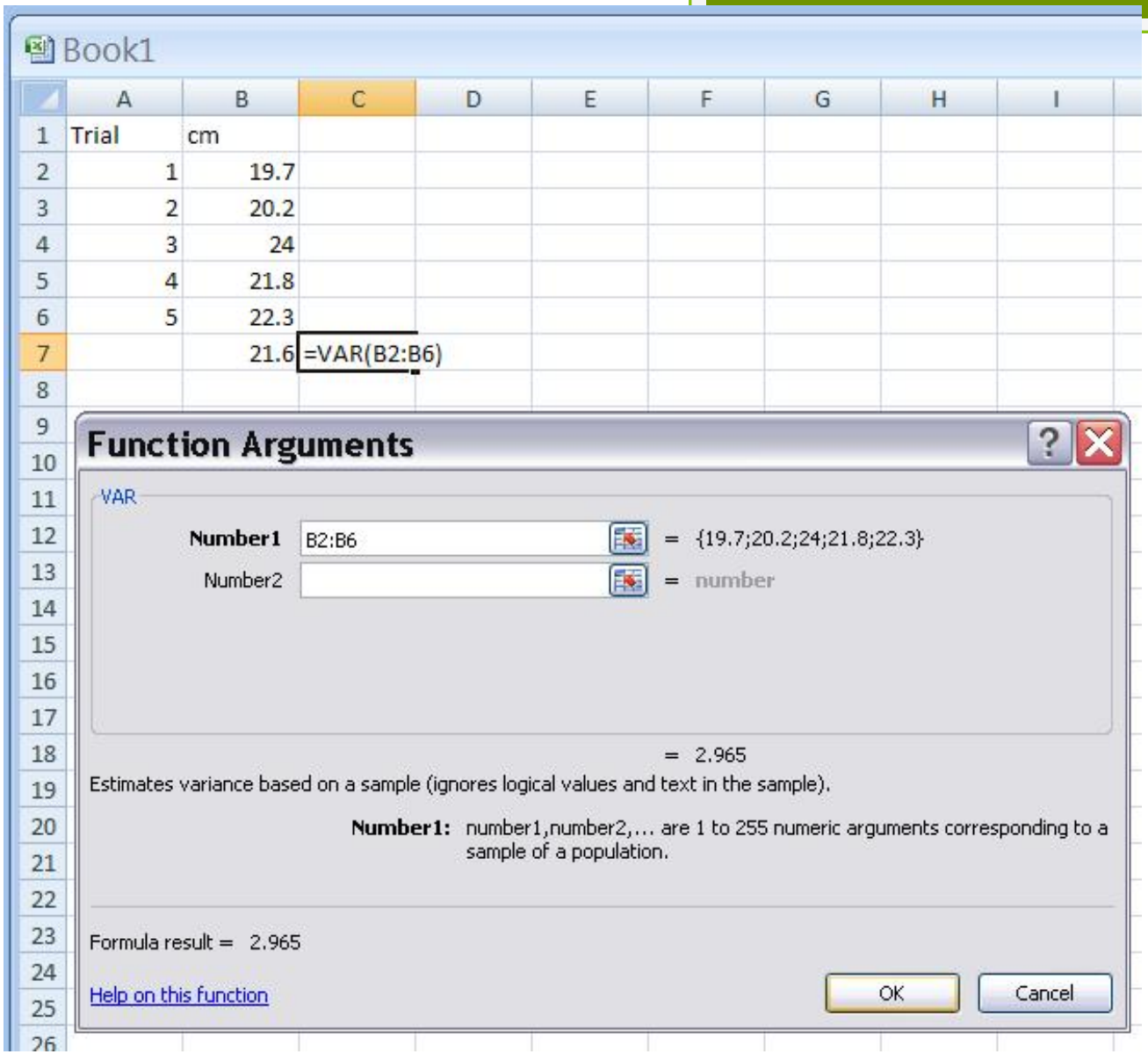
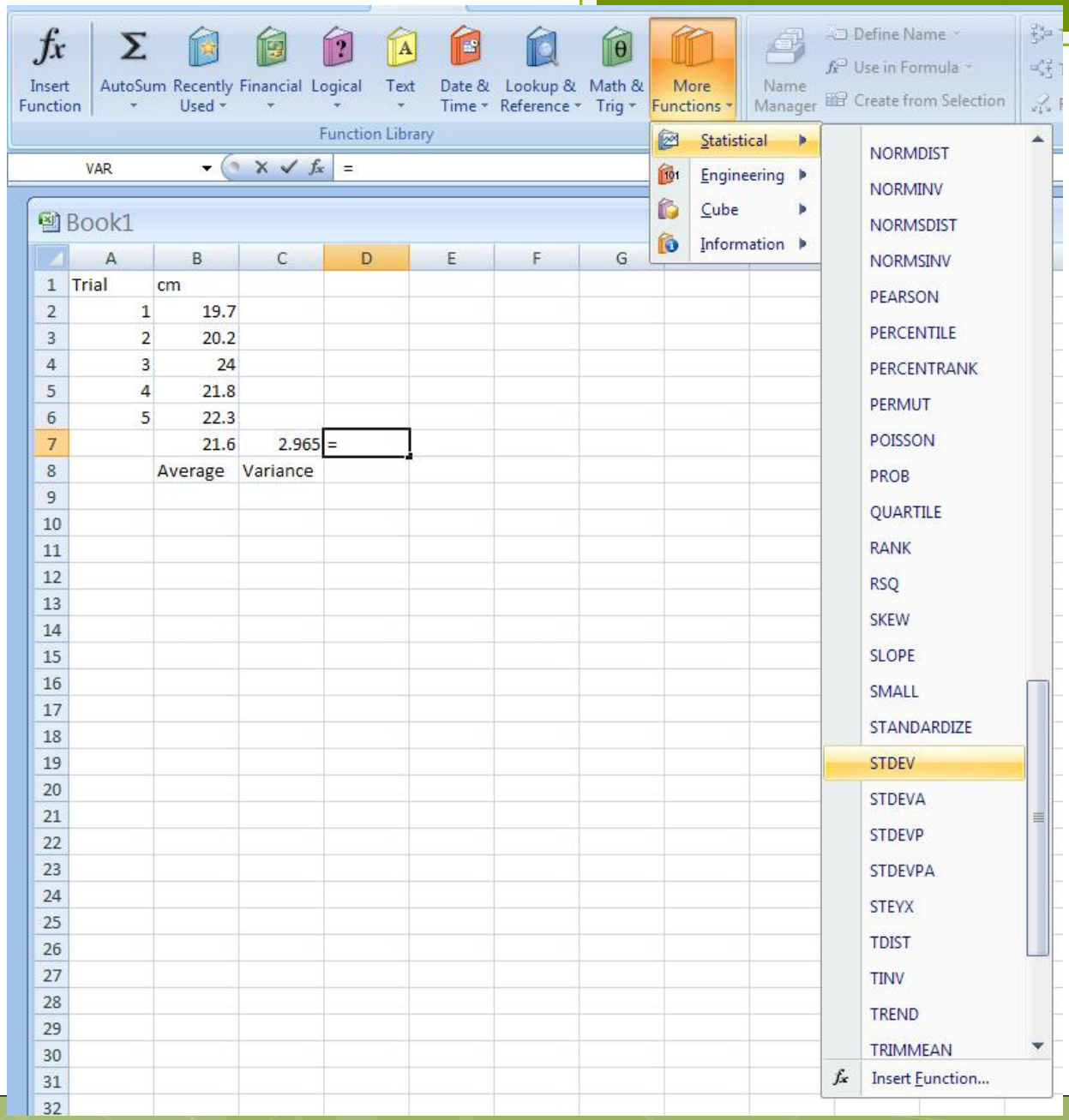
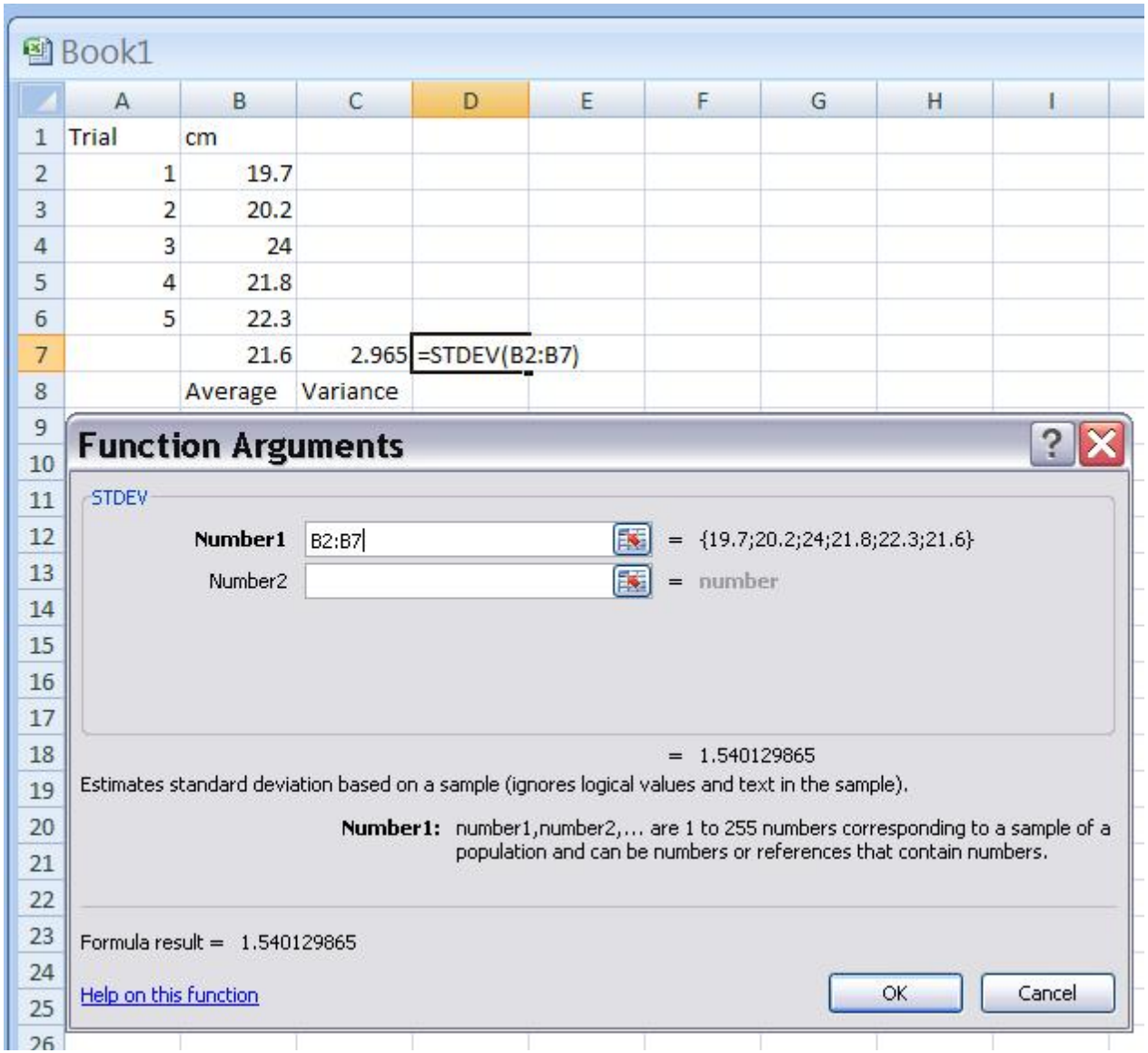
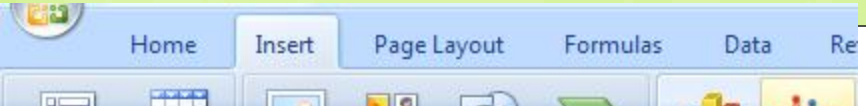
PROB
QUARTILE
RANK
RSQ
SKEW
SLOPE
SMALL
STANDARDIZE
STDEV
STDEVA
STDEVP
STDEVPA
STEYX
TDIST
TINV
TREND
TRIMMEAN
TTEST
VAR
VARA
VARP
VARPA
WEIBULL
ZTEST
Insert Function...
Trial
cm
1 19.7
2 20.2
3 24
4 21.8
5 22.3
21.6 =


Book1
A B C D E F G H I
1 Trial cm
2 1 19.7
3 2 20.2
4 3 24
5 4 21.8
6 5 22.3
7 21.6 =VAR(B2:B6)
Function Arguments
VAR
Number1 B2:B6 = {19.7;20.2;24;21.8;22.3}
Number2 = number
= 2.965
Estimates variance based on a sample (ignores logical values and text in the sample).
Number1: number1,number2,... are 1 to 255 numeric arguments corresponding to a sample of a population.
Formula result = 2.965
Help on this function
OK
Cancel



	A	B	C	D
1	Trial	cm		
2	1	19.7		
3	2	20.2		
4	3	24		
5	4	21.8		
6	5	22.3		
7		21.6	2.965	=
8		Average	Variance	


Book1
A B C D E F G H I
1 Trial cm
2 1 19.7
3 2 20.2
4 3 24
5 4 21.8
6 5 22.3
7 21.6 2.965 =STDEV(B2:B7)
8 Average Variance
Function Arguments
STDEV
Number1 B2:B7 = {19.7;20.2;24;21.8;22.3;21.6}
Number2 = number
= 1.540129865
Estimates standard deviation based on a sample (ignores logical values and text in the sample).
Number1: number1,number2,... are 1 to 255 numbers corresponding to a sample of a population and can be numbers or references that contain numbers.
Formula result = 1.540129865
Help on this function
OK
Cancel



B1 | cm

Book1

	A	B	C	D	E
1	Trial	cm	Mean		
2	1	19.7	21.6		
3	2	20.2	21.6		
4	3	24	21.6		
5	4	21.8	21.6		
6	5	22.3	21.6		
7		21.6	2.965	1.54013	
8		Average	Variance	StDev	
9					

Book1

	A	B	C	D	E	F	G	H	I
1	Trial	cm	Mean						
2	1	19.7	21.6						
3	2	20.2	21.6						
4	3	24	21.6						
5	4	21.8	21.6						
6	5	22.3	21.6						
7		21.6	2.965	1.54013					
8		Average	Variance	StDev					
9									
10									
11									
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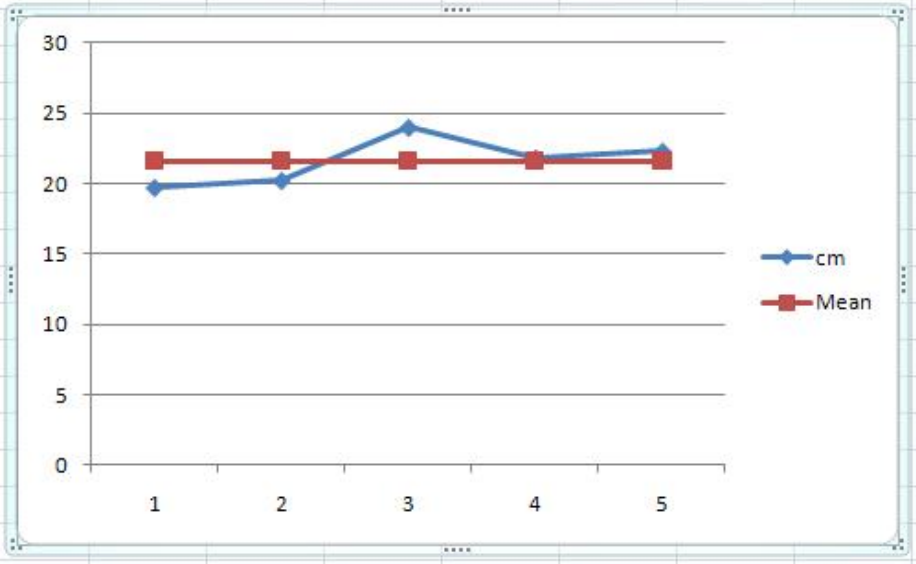
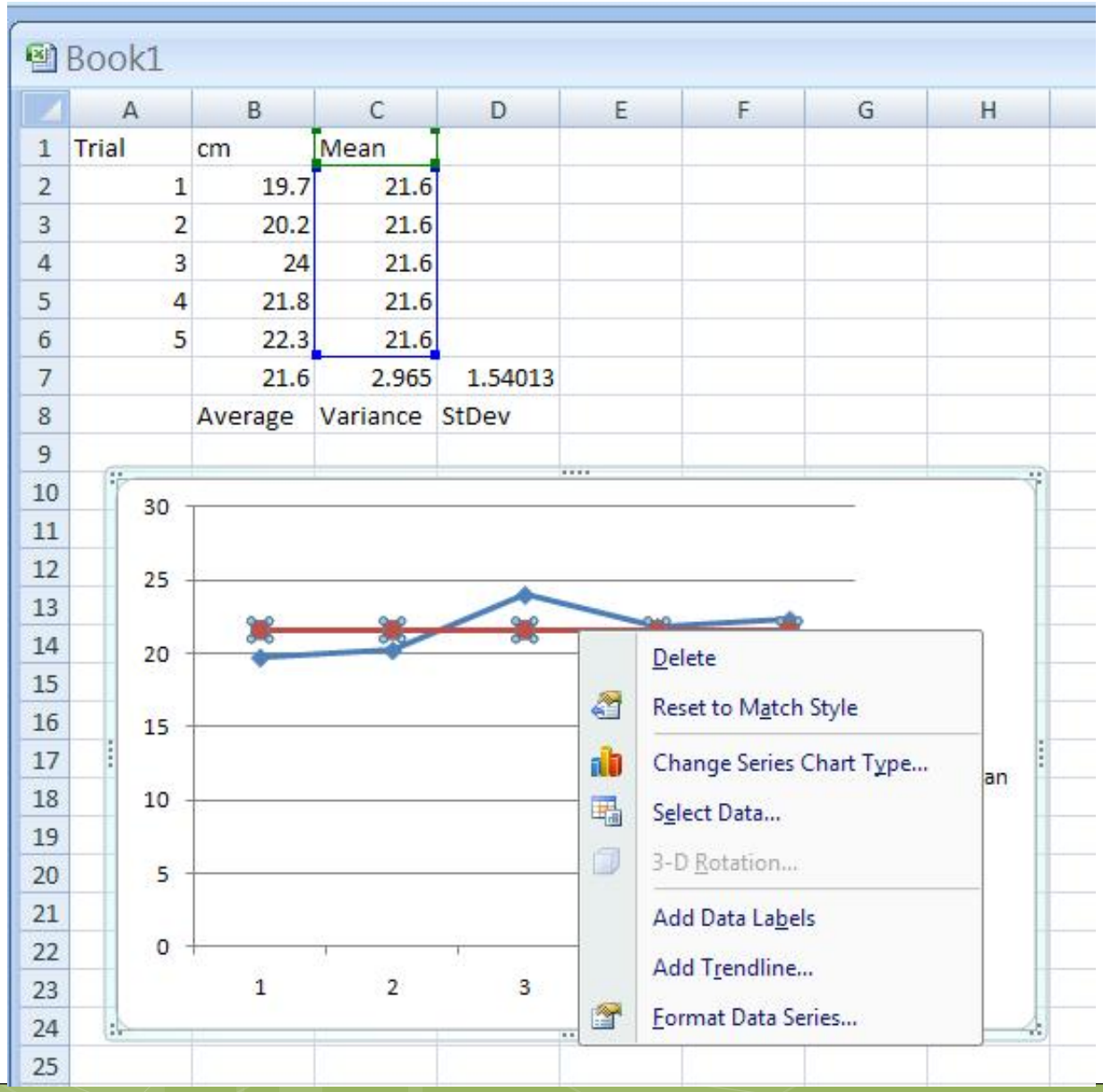
26									




Book1
Trial
cm
Mean
1
19.7
21.6
2
20.2
21.6
3
24
21.6
4
21.8
21.6
5
22.3
21.6
21.6
2.965
1.54013
Average
Variance
StDev
30
25
20
15
10
5
0
1
2
3
Delete
Reset to Match Style
Change Series Chart Type...
Select Data...
3-D Rotation...
Add Data Labels
Add Trendline...
Format Data Series...

Book1

	A	B	C	D
1	Trial	cm	Mean	
2	1	19.7	21.6	
3	2	20.2	21.6	
4	3	24	21.6	
5	4	21.8	21.6	
6	5	22.3	21.6	
7		21.6	2.965	1.54013
8		Average	Variance	StDev